The Journal of Laboratory and Clinical Medicine

VOL. 29 OCTOBER, 1944 NO. 10

PROGRESS

THE PROGNOSTIC SIGNIFICANCE OF AN ELEVATED BLOOD CREATININE

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IT IS now thirty years since it was first observed that the creatinine of the blood is markedly elevated in cases showing severe impairment of renal function. Since the initial observations were made a number of controversies have arisen involving not only the validity of the determination itself but also the interpretation which can justly be placed upon the results obtained. It is believed that we now possess sufficient facts and a sufficiently long perspective to adequately evaluate the accuracy and clinical importance of this determination.

Before entering upon a discussion of the creatinine of the blood it may be well to indicate some of the most fundamental facts regarding creatine-creatinine metabolism. Folin[1] was the first to show that the amount of creatinine excreted in the urine by a normal individual on a meat-free diet is quite independent of either the amount of protein in the food or the total nitrogen in the urine, the amount excreted from day to day being practically constant, thus pointing conclusively to its endogenous origin. Creatinine is the anhydride of creatine and one might assume on *a priori* grounds that creatine is the precursor of creatinine. However, this was questioned by no less an authority than Folin himself. The origin of creatine was even more obscure, although logic and some experimental data suggested that arginine might be the precursor. Very recently Borsook,[2] du Vigneaud,[3] Schoenheimer,[4] and their coworkers have definitely answered the question regarding the origin of creatine, and have conclusively proved that creatinine originates from creatine. du Vigneaud and Schoenheimer were able to arrive at their conclusions by the use of tracer isotopes. The im-

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Received for publication, July 30, 1944.

mediate precursor of creatine is glycocyamine. This is formed from glycine and arginine (also citrulline) in the kidney, while glycocyamine is methylated in the liver by methionine (or a derivative of methionine) to form creatine.

The importance and significance of muscle creatine was not appreciated until Fiske and Subbarow[5] in Folin's laboratory in 1927 showed that in living muscle creatine is present very largely in combination with phosphoric acid, probably as a potassium salt. Quite as Folin and Denis[6] prophesied in 1914, creatine has been found to be a post-mortem product. In the process of fatigue and death phosphocreatine (or creatine phosphate) breaks up into its constituent parts. Although the complete story of the energetics of muscular activity has not been told, it is evident that creatine phosphate and other compounds of phosphoric acid are vitally concerned in the production and transfer of energy. Creatine is a necessary link in this chain. In man the normal creatine concentration of voluntary muscle is about 440 mg. per 100 grams of muscle, less than half this concentration (about 200 mg.) in the left ventricle of the heart, still less in the brain (about 170 mg. in the cerebellum and 110 mg. in the cerebrum), and much less in smooth muscle (about 60 mg.).

CREATININE CONTENT OF THE BLOOD

Folin and Denis[7] were the first to present any very extensive data on the creatinine content of the blood, although almost simultaneously Neubauer[8] reported an observation on one case of "uremia," while Myers and Fine[9] presented several analyses on two cases of nephritis showing marked retention of creatinine. Folin and Denis gave observations on nine cases of "uremia" and were the only observers to give figures for normal human blood. Shaffer[10] had begun a study of this question some years previously and had made a preliminary report with Reinoso,[11] but unfortunately did not give his protocols until after the appearance of the paper by Folin and Denis.

Normal Creatinine Content of Blood.—Nearly all the original analyses were made on whole blood after precipitating the proteins with pieric acid. The Jaffé color reaction was carried out directly on the blood filtrate by the simple expedient of adding 0.5 c.c. of 10 per cent sodium hydroxide to 10 c.c. of the saturated pieric acid filtrate. With this procedure creatinine values between 1 and 2 mg. per 100 c.c. are obtained for normal individuals, being nearer 1 than 2 mg. As soon as one passes to hospital patients, however, slightly higher values may be encountered, especially if whole blood is employed for analysis. Although the great majority of cases without renal involvement show creatinine figures on the whole blood below 2.5 mg. per 100 c.c., very occasionally figures as high as 3.5 mg. may be encountered that are not readily explained.

Although most of the earlier analyses were made on whole blood by the Folin method[12] or the Myers and Fine modification,[13] it was later shown by Hunter and Campbell[14] that there is present in the corpuscles a substance which interfered, and tended to raise materially the values obtained on whole blood. Obviously the percentage of error here would be greatest in normal blood with a low concentration of creatinine. On this account these authors recommended the use of plasma or serum. Later methods employing other protein precipitants and a lower concentration of pieric acid, thus making the Jaffé color reaction more specific for creatinine, tended to lower the values obtained on normal blood.

Consequently it would appear that strictly normal human plasma contains between 0.5 and 1.5 mg. creatinine per 100 c.c. with an average of close to 1.0 mg.* Folin and Svedberg[15] obtained somewhat similar figures on Folin's "unlaked blood" filtrate.

Before discussing the clinical significance of abnormal amounts of creatinine in blood it may be well to discuss briefly the question that has been raised as to whether or not creatinine is actually present in blood.

Controversy on the Presence of Creatinine in Blood.—In 1922 Behre and Benedict[16] presented evidence to show that creatinine does not exist in normal blood. The facts upon which they made this deduction were: (1) that creatinine can be removed from blood by bone black, but bone black does not affect the chromogenic substance present in blood filtrates, which reacts with picric acid and sodium hydroxide; (2) that a substance present in picric acid blood filtrates gives a good reaction with sodium carbonate, and this reaction is increased in bloods showing an abnormally high "creatinine" content, whereas pure creatinine in picric acid is only slightly affected by carbonates; (3) that creatinine is readily destroyed by heating in alkaline solution, but the chromogenic substance present in blood is not appreciably affected; (4) that kaolin completely removes small amounts of creatinine from blood, but does not affect the picric acid-alkali reacting substance. Their observations were confirmed, and two additional observations support this view: (1) Benedict and Behre[17] found that the color obtained on blood filtrates and dilute serum ultrafiltrates with 3, 5-dinitrobenzoate differs from that of creatinine, and (2) the apparent creatinine of normal dog serum ultrafiltrates (Gaebler[18]) and plasma filtrates of human and other species (Behre and Benedict[19]) fail to precipitate under conditions which precipitate added creatinine. Shortly after the appearance of Behre and Benedict's original paper, Folin[20] commented: "In view of the many still active investigators who in the past have made contributions to the creatine-creatinine problem one can safely predict that the findings and conclusions of Behre and Benedict will not remain without contradiction or verification."

Quite as Folin prophesied the observations and interpretations of Behre and Benedict led to a bitter controversy. There is no question about the presence of creatinine in urine. It would appear therefore that the kidney must (1) either separate and concentrate creatinine present in blood, or (2) form creatinine from a closely related precursor compound present in the blood, and then excrete it. If the observations of Behre and Benedict are correct, the latter would serve to explain the discrepancies. The opponents of Behre and Benedict, however, feel that this explanation is unnecessary. Hayman, Johnston and Bender[21] found that when trichloroacetic acid (rather than picric acid) was employed as the protein precipitant on serum or plasma, the filtrate differed little in its reaction from pure creatinine. Danielson[22] came to much the same

*Arkin, Popper and Goldberg[24] have recently recommended the determination of plasma creatinine as a test of low grade kidney damage. They consider the normal range to lie between 0.60 and 0.95 mg. per 100 c.c., and regard any increase over 1.0 mg. as a probable sign of impaired glomerular filtration. They feel that the test in the low ranges is superior to the determination of the nonprotein nitrogen and urea, not only for theoretical reasons (lack of reabsorption in the tubules) but also because of the sharper limits between the normal and pathological range, and consequently provides a helpful diagnostic procedure for the detection of low grade kidney damage. The test is regarded as of diagnostic and prognostic importance in acute nephritis, chronic nephritis, nephrosis, nephrosclerosis, urologic conditions and heart failure.

conclusion from a study of plasma ultrafiltrates. With the aid of a specific bacterial enzyme, Miller and Dubos[23] have approached the problem in a different way and conclude that in normal individuals creatinine constitutes 80 to 100 per cent of the chromogenic material in serum or plasma.

Gaebler in particular has made important contributions to a solution of this problem. With Keltch[24] and independently[25] he demonstrated that creatinine could be isolated from normal blood, and in quantity from retention blood. The creatinine thus isolated was definitely identified chemically. Although originally opposing Behre and Benedict, he[25] later agreed that creatinine as such was not present in detectable amounts in normal blood. More recently Gaebler[26] and Gaebler and Abbott[27] have shown that most of the apparent creatinine of ultrafiltrates of normal blood can be precipitated with picric acid and rubidium. Goudsmit[28] has compared the apparent creatinine content of renal venous blood and of arterial blood, and found the former consistently lower, thus definitely indicating that the apparent creatinine of the blood is the precursor substance of urinary creatinine.

It is evident therefore that the apparent creatinine of blood, whether creatinine itself or a precursor, can be converted in the test tube, in considerable part at least, to creatinine and can be so converted by the kidney. Consequently the apparent creatinine of blood, if not creatinine, has the same significance in blood as creatinine.

CREATININE CONTENT OF THE BLOOD IN DISEASE

In general it may be stated that the concentration of creatinine in the blood (plasma) is not significantly altered in disease, except where marked impairment in renal function has occurred. While normal renal function is primarily dependent upon normal kidneys, it is also dependent upon normal blood flow to the kidneys. This may be sufficiently impaired in marked dehydration and severe heart disease to have a definite influence on the level of the nitrogenous waste products in the blood, including creatinine. Practically it has been found that there is no indication for making a blood creatinine determination until after the level of the blood urea has exceeded the upper normal limits.

It should be noted that the older methods on whole blood have occasionally yielded moderately elevated creatinine values (figures between 3 and 4 mg. per 100 c.c.) in syphilis, in certain heart conditions, sometimes in fevers, such as pneumonia, in advanced malignancies, and in some cases of severe diabetes. It is well known that an increased formation of creatinine occurs in fevers, while in diabetes the influence of the high glucose concentration on the Jaffé color reaction for creatinine accounts in part for the elevated values in some cases.

High values for the creatinine of the blood (over 4 mg. per 100 c.c.) have been observed in a variety of conditions in which renal function is seriously impaired. The following conditions may be mentioned: various forms of Bright's disease, but chiefly glomerulonephritis and nephrosclerosis, pyelonephritis, bichloride poisoning, double polycystic kidney, prostatic hypertrophy, vesical neoplasms, intestinal obstruction, acute suppression of urine from various causes, etc.

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Prognostic Value of the Blood Creatinine.—Attention was apparently first called to the unfavorable prognostic import of an elevated blood creatinine by Myers and Lough[29] in 1915. They suggested that since creatinine was almost wholly endogenous in origin and, furthermore, since it was apparently the most readily eliminated of the three nitrogenous waste products, uric acid, urea and creatinine, it should be of greater prognostic value than either uric acid or urea. A year later Chace and Myers[30] further discussed this subject and stated on the basis of the generally accepted average figures for the concentration of these three constituents in the blood and urine, that whereas the kidney normally concentrates the uric acid about 20 times, and the urea about 80 times, it concentrates the creatinine about 100 times. More recent studies by Mayrs,[31] Rehberg,[32] MacKay and Cockrill,[33] and Cope,[34] dealing with the concentration ratios of administered creatinine and urea, definitely showed that the kidney is able to concentrate creatinine to a greater extent than urea, and thus bear out the original contention of Myers and his coworkers. In this connection some observations of Berglund and Frisk[35] are also of interest. They have compared the ability of the kidney to excrete creatinine with uric acid. They state: "Whether measured directly or under the influence of drugs . . . the efficacy of uric acid elimination is greatly inferior to the elimination of creatinine. Uninfluenced by drugs the elimination is a fifth to an eighth only as efficacious as the mechanism for creatinine elimination."

Myers and Lough[29] stated in their original paper: "The creatinine rises above 2.5 mg. per 100 c.c. of blood almost without exception only in conditions with renal involvement. Creatinine values from 2.5 to 3.0 mg. may be viewed with suspicion; figures from 3.0 to 5.0 mg. regard as decidedly unfavorable, while over 5.0 mg. probably indicates an early fatal termination." Although with improvements in methods the values obtained for the creatinine of the blood have tended to fall slightly, the above statement, made nearly thirty years ago, would still appear to stand.

In 1919 Myers and Killian[36] again discussed the prognostic value of the creatinine of the blood in nephritis on the basis of 100 cases with over 4 mg., and 85 cases with over 5 mg. creatinine per 100 c.c. of blood (5-33 mg.), studied between March, 1914 and January, 1919. Of the 85 cases, 80 were dead at the time of their publication, 3 died shortly afterwards and 2 recovered. The 2 cases which recovered showed only a temporary elevation of the creatinine above the 5 mg. level. In this connection Myers and Killian commented: "In such conditions as bichloride poisoning and acute nephritis it is possible to have considerable nitrogen retention with ultimate recovery, for the reason that here the activity of the kidney is only temporarily depressed." The authors noted that a considerable number of the 85 cases were able to be up and about and some showed decided clinical improvement. They felt that the following of the blood creatinine gave them a better prognostic insight into these cases than either the blood urea or the phenolsulfonphthalein tests which were done simultaneously.

Case E. M. is a good illustration of a patient in the last stages of the disease, but who, nevertheless, was able to be up and about (see Table I).

Case 2. J. B. illustrates the prognostic value of the blood creatinine particularly well. This patient's blood showed a creatinine of 7.5 mg. when he first

TABLE I

SERIAL OBSERVATIONS ON FOUR CASES ILLUSTRATING THE PROGNOSTIC VALUE OF THE BLOOD CREATININE

CASE	DATE	CHEMICAL BLOOD ANALYSES			
		CREATININE	UREA N	URIC ACID	CO_2 CAPACITY
		mg.	mg.	mg.	c.c.
	1915				
1. E. M.*	Nov. 30	17.5	97	6.6	24
	Dec. 4	21.5	129	6.4	21
	Dec. 10	22.3	132	7.0	26
	Dec. 17	24.2	150	5.6	15
	Dec. 21	28.6	186	5.0	18
	Dec. 24	26.7	200	7.7	12
	1916				
2. J. B.†	Dec. 22	7.5	60		32
	1917				
	Jan. 5	9.7	135		
	Jan. 9	12.5	110		49
	Jan. 16	9.5	93		
	Jan. 30	7.8	48	5.00	
	Feb. 13	7.8	51	6.4	51
	Feb. 27	7.5	45	7.1	
	Mar. 6	6.4	49	6.1	
	Mar. 20	10.6	53		
	Apr. 3	7.2	49		
	Apr. 13	6.4	46	5.9	
	Apr. 27	7.5	45		
	May 15	7.3	33		
	May 22	7.3	24		
	June 5	6.7	34		
	July 17	6.8	29		
	1921				
3. C. W.‡	Jan. 17	11.0	90		
	Jan. 26	11.1	111		
	Feb. 16	6.0	40		37
	Feb. 23	6.8	32		37
	Mar. 2	5.3	31		
	Mar. 10	4.9	35		28
	Mar. 18	4.6	37		44
	Apr. 13	7.1	49	4.8	31
	Nov. 4	5.1	41	6.5	35
	1922				
	Jan. 31	4.2	44	10.1	37
	Feb. 11	4.1	38	5.2	38
	1923				
	Sept. 4	9.2	60	5.3	
	1917				
4. J. C.§	Apr. 24	3.8	28	6.8	50
	May 8	6.1	41	5.5	
	May 15	5.2	30	7.7	
	May 22	3.3	28	7.7	
	June 1	3.1	36	10.4	
	June 8	4.5	26		
	1918				
	Apr. 2	3.8	57		
	Apr. 26	4.5	51	6.7	
	1919				
	Jan. 19	4.3	64		34
	1920				
	Feb. 18	7.2	56	5.0	

*1. E. M., male, aged 39 was up and about on Nov. 30, did not realize the seriousness of his condition, had stopped in New York on his way to Florida for the winter; systolic blood pressure 240 mm. Hg; 'phthalein output 0 on three examinations; sp. gr. of urine continuously 1.005 on Dec. 10; died on Dec. 25.

†2. J. B., male, aged 34 kept on low protein diet for five months; discharged from hospital clinically improved on June 9; returned to work as guard on subway; died Nov. 7, 1917.

‡3. Col. C. W., male, aged 55, enlarged prostate and chronic nephritis, cystotomy Jan. 19, and prostatectomy Mar. 25, 1921; 'phthalein output Feb. 11, 1922 5 per cent; died Oct. 11, 1923.

§4. J. C., male, aged 60 continued active during most of this period as club manager, although he spent a number of rest periods in hospital beginning in April 1916; 'phthalein output 7 per cent on May 22, 1917; died in June 1920.

came under observation, and although he showed considerable clinical improvement over a period of nearly seven months, still his blood creatinine remained constant, except for a temporary elevation to 12.5 mg. The dietetic measures which were employed to alleviate the nitrogen retention resulted in a gradual reduction in the urea nitrogen from 135 mg. per 100 c.c. to a low of 24 mg. Despite his high creatinine (last determination 6.8 mg.), the patient left the hospital feeling quite well, returned to work as a guard on the subway and did not die until about five months later.

The prognostic value of the creatinine is likewise well illustrated by the data on *Case 3 C. W.* (Table I), a case of chronic nephritis complicated by prostatic hypertrophy. On admission the creatinine was 11.0 mg., but 13 months later after a prostatectomy had dropped to a low of 4.1 mg. Six weeks after cystotomy the urea N dropped from a high of 111 to a low of 31 mg. The temporary increased elevation of both the blood creatinine and urea N following both surgical operations is of interest. Although this patient remained quite active for about two years following his prostatectomy, the ultimate outcome was clearly indicated by the blood creatinine, and he died about 2 years and 9 months after first admission to the hospital.

Since cases that recover, or live for some months, after the creatinine of the blood has exceeded 5 mg. per 100 c.c. are exceptional, such cases merit special consideration. The individual observations on *Case 1. J. C.* (Table I) are of interest in this connection. This case remained nearly stationary for a longer period than any other in the series. On his last admission to the hospital the patient was definitely worse and the creatinine had risen to 7.2 mg. He died about four months after leaving the hospital. Even among cases having very high blood creatinine there were many who were able to be up and about and some who showed considerable clinical improvement. It was in these cases that the blood creatinine gave a particularly good prognostic insight into the true nature of the condition.

A study of 1600 (about 1300 additional) creatinine determinations on cases with values above 4 mg., up to January, 1929, did not lead Myers and Killian to alter the view expressed 10 years earlier.

In the author's book on the chemistry of the blood[37] a table is presented giving observations on seven cases of intestinal obstruction made by Killian in 1921. It is of interest to note that, of the seven cases the five who died, all had blood creatinine above 5 mg. per 100 c.c., the range being from 5.2 to 17.0 mg. The two cases which recovered showed maximum creatinine values of 2.2 and 4.7 mg. respectively.

Comparing the creatinine and urea, Myers and Killian[36] state: "Theoretically, the amount of the increase of the creatinine of the blood should be a safer index of the decrease in the permeability of the kidney than the urea, for the reason that creatinine on a meat-free diet is entirely endogenous in origin and its formation (and elimination normally) very constant. Urea on the other hand, is largely exogenous under normal conditions and its formation consequently subject to greater fluctuation. For this reason it must be evident that a lowered nitrogen intake may reduce the work of the kidney in eliminating urea, but cannot affect the creatinine to any extent. Apparently the kidney

is never able to overcome the handicap of a high creatinine accumulation. It would seem that creatinine, being almost exclusively of endogenous origin, furnishes a most satisfactory criterion as to the deficiency in the excretory power of the kidneys, and a most reliable means of following the terminal course of the disease, though it should be noted that urea, being largely of exogenous origin, is more readily influenced by dietary changes, and therefore constitutes a more sensitive index of the response to treatment."

Another factor which may play a role in the greater prognostic significance of an elevation of the blood creatinine than the blood urea is the difference in the mechanism by which these two constituents are eliminated by the kidney.[38] Urea is apparently eliminated entirely by glomerular filtration and tubular reabsorption of water, although there is appreciable back diffusion owing to the great diffusibility of urea. While the chief factor concerned in the excretion of creatinine is again glomerular filtration and tubular reabsorption of water, there is not only no back diffusion but also the tubules of the human kidney have the ability to add (secrete) an appreciable amount of creatinine to the glomerular filtrate.

The reliability of elevated blood creatinine values as an unfavorable prognostic sign in terminal renal insufficiency was quickly and amply confirmed by workers in various parts of the world. Among these may be mentioned Rosenberg[39] Feigl,[40] Rabinowitch,[41] Moreau and Diamant,[42] Jeanbrau and Cristol,[43] Feinblatt,[44] Hubbard,[45] Cantarow and Davis,[46] Warter,[47] and Popper, Mandel and Mayer.[48] Although most workers have supported fully the conclusions of Myers and Lough,[29] a few have felt that the creatinine gives little or no more information than the blood urea. Peters and Van Slyke,[49] in particular have emphasized this point although admitting that the "gravity of a high blood creatinine above 5 mg. per 100 c.c., a nonprotein nitrogen above 100 mg., or a urea nitrogen above 80 mg. in the blood is of serious import. Usually creatinine and urea rise together but when they do not the creatinine may be either the first or last to indicate the approach of uremia." Hubbard[45] likewise observed that the "urea and creatinine concentrations in the blood parallel each other." He felt that sudden changes in blood urea concentration were probably due more to variations in the rate of urea formation, than to differences in the permeability of the kidney for these two compounds.

However, this has not been the general conclusion of most workers in this field. Patch and Rabinowitch[50] state in this connection: "A large series of observations led to the finding of cases in which the general relationships between the urea and creatinine concentrations of blood were absent. In these cases, high values were found associated with normal or nearly normal values. . . . It was found that, in spite of high urea values, symptoms of uremia were usually absent when the creatinine values were normal or nearly normal. It was also found that though high creatinine values and symptoms of uremia were accompanied by positive diazo color reactions, this was not necessarily so with high urea values. . . . The clinical value of these observations, from the point of view of the chemical reactions of the blood, lies in their showing that urea studies unaccompanied by observations on 'creatinine' and the diazo color reaction should not be relied on in estimating progress or prognosis."

Most observers have noted a lack of relation between the levels of creatinine and urea in cases of renal disease, the chief exception being cases of anuria, e.g., bichloride poisoning. Obviously in such cases both creatinine and urea are being simultaneously and completely retained. The situation is different, however, in cases of nephrosclerosis, chronic glomerulonephritis, intestinal obstruction and the renal complications associated with prostatic hypertrophy and neoplasms of the bladder. In the last mentioned conditions there may be oftentimes a disproportionate increase in the blood urea. Some years ago in following cases at the Cleveland City Hospital in cooperation with Dr. R. O. Bowman and Miss Miriam Dice, it appeared that we had made an interesting observation bearing on this point. It was observed that cases dying from chronic glomerulonephritis showed much higher blood urea values than cases dying with nephrosclerosis with essentially the same values for creatinine, i.e., whereas a case of chronic glomerulonephritis with a blood creatinine of 5 to 10 mg. might have a blood urea nitrogen of 200, a case of nephrosclerosis, with the same range of blood creatinine values, would show a urea nitrogen of only 50 to 70 mg. However, on studying further cases (all diagnoses were confirmed by autopsies) it was found that the above statements were true in only 65 to 75 per cent of the cases.

The Van Slyke urea clearance test is not only an exceedingly valuable diagnostic test of renal insufficiency, but furnishes very valuable prognostic information as well. In this connection, Van Slyke et al.[51] conclude: "In patients with diminished renal function the blood urea clearance shows evidence of the diminution sooner than does the blood creatinine content, the blood urea content without relation to the urea excretion, or the phenolsulfonphthalein excretion. The blood urea clearance usually falls below 50 per cent of its normal value before any of the other three values show any abnormality. Only after the blood urea clearance indicates less than 20 per cent of normal renal function are all values for blood urea and creatinine content, and for phenolsulfonphthalein excretion, found outside the limits of normal variation." Bruger and Mosenthal[52] have also compared the blood creatinine with the urea clearance test and state: "It has been appreciated for a long time that creatinine rises in the blood only when renal function becomes markedly impaired (Chace and Myers, Myers, Fine and Lough, and Myers and Lough). Our results show that normal creatinine values may be found in the blood with as much as 85 per cent of kidney function lost, as measured by the urea clearance test. When the urea clearance has fallen to about 5 per cent of normal and uremia is impending, the creatinine begins to mount in the blood."

High Creatinine Values in Acute Conditions.—Not long after the original publication of Myers and Lough[29] in 1915 a number of different workers observed that a blood creatinine in excess of 5 mg. per 100 c.c. did not possess the same unfavorable significance in acute conditions as in chronic nephritis, and presented reports on survival cases, the largest number being cases of bichloride poisoning. Myers and Killian[36] reported two acute cases with creatinines of 6.1 and 5.6 mg. respectively with recovery. They also called attention to the fact that occasionally in the acute exacerbation of chronic nephritis the creatinine may exceed 5 mg. for a short period, then decline below this level, rising again

above this level before fatal termination. Occasionally this may occur several times in the course of the disease.

Non-fatal cases of mercury bichloride poisoning with high creatinine values have been reported as follows: Cohen and Bernhard[53] 9.3 mg., Campbell[54] 12.5 mg., Killian[55] two cases, 12.0 and 15.2 mg. respectively, Gatewood and Byfield[56] 11.3 mg., Muntwyler, Way and Pomerene[57] two cases, 12.0 and 15.5 mg. respectively and Bowman and Wolpaw[58] a case of uremia, apparently due to mercuric chloride, 24.3 mg. per 100 c.c. Although recovery in such cases appears to be the exception, rather than the rule, the cases cited indicate that while more than 5 mg. creatinine per 100 c.c. of blood furnishes a grave prognosis in poisoning with mercuric chloride, the prognosis need not necessarily be hopeless.

TABLE II

CASE (L. M.) WITH ANURIA WITH VERY HIGH CREATININE AND RECOVERY[59]

DATE 1922	CHEMICAL BLOOD ANALYSES			
	CREATININE	UREA N	URIC ACID	CO_2 CAPACITY
	mg.	mg.	mg.	c.c.
Mar. 13		12.1	4.6	
Mar. 27	12.5	62.5		50
Mar. 30	17.0	83.3		32
Mar. 31	23.1	92.0		21
Apr. 2	20.4	98.6		56
Apr. 3	20.0	82.0		82
Apr. 4	21.2	104.0		63
Apr. 10	9.2	56.6		56
Apr. 15	4.4	48.0		63
Apr. 22	3.2	12.9		61
May 4	2.7	9.7		56
July 11		16.0	4.2	

Nephrolithiasis; patient developed anuria following a unilateral pyelogram and displacement of renal calculus; alkali therapy Mar. 31 to Apr. 3, forced fluids through duodenal tube from Mar. 31; passed calculus Apr. 8; discharged May 15.

The highest blood creatinine we have observed with ultimate recovery is given in Table II. This patient (L. M.) developed anuria following an unilateral pyelogram and displacement of renal calculus.[59] After the blood creatinine had risen to 23.1 mg. and the CO_2 combining power of the plasma had fallen to 21, alkali therapy was started and fluid forced with the aid of a duodenal tube, about 1,000 to 4,000 c.c. of fluid being given daily. It is believed that in this case the blood analyses gave a clear understanding of the condition without which the patient unquestionably would have died.

Selman and Linegar[60] have reported a very interesting case in which the creatinine rose to 15.4 mg. and the urea nitrogen to 142 mg. per 100 c.c. with ultimate recovery. The patient suffered from severe asthma and shock, the blood pressure being too low to measure with a mercury manometer. The authors suggest the resultant disturbance in blood flow to the kidney as the probable cause of the elevation of the nitrogenous metabolites in the blood. It took about eight days for the blood accumulation to reach its peak, while twelve days were required for its return to normal.

Kilduffe[61] in discussing a case which remained alive for fifteen months with blood creatinine values ranging from 10 to 20 mg. per 100 c.c., made the

following comment regarding recovery cases: "Hypercreatininemia may, with two exceptions, be accepted as of ominous import, the observations of many indicating that a blood creatinine of more than 5 mg. per cent is the rather immediate forerunner of a fatal termination. The exceptions to this clinical rule are: (1) anuria, in which, if the cause can be found and removed, creatinine retention is not of prognostic import, and (2) acute nephritis in which temporary high values may be encountered. With these two exceptions hypercreatininemia always signifies an extreme degree of functional renal insufficiency, the kidney apparently being unable to recover from the damage thus indicated. . . . The usual interpretation regards creatinine values of 2.5 to 3 mg. per cent as suspicious; 3 to 5 mg. per cent as unfavorable; and over 5 mg. per cent as indicative of an early fatal termination. While occasional recoveries in the presence of hypercreatininemia have been reported, these are so exceptional as not to invalidate the general rule."

COMPARISON OF CREATININE WITH OTHER PROGNOSTIC TESTS

In addition to the blood urea, and Van Slyke urea clearance tests already mentioned, and the fact that a sudden and pronounced upturn in the blood uric acid may be an unfavorable prognostic sign several other tests have been suggested which have a grave prognostic omen. These are an elevation of the serum inorganic phosphorus, increases in the amount of indican and strongly positive diazo and xanthoproteic reactions. Most of these become positive slightly later than the creatinine, and therefore are even more grave signs of impending death. However, it appears doubtful if they are any more reliable than the creatinine, and the fact that they become definitely positive a little later can hardly be considered an advantage. Nevertheless, they are excellent confirmatory tests.

Inorganic Phosphorus.—de Wesselow[62] in particular has emphasized the unfavorable prognostic value of an elevated inorganic phosphorus in nephritis, especially when associated with uremia. He points out that on the average the retention of phosphates and urea run a parallel course, but observes that a confident prognosis can scarcely be ventured upon the estimation of the blood urea alone. He is of the opinion that although nitrogen retention is a valuable prognostic index of the severity of nephritis, nitrogen retention per se is harmless. His special reason for suggesting the use of the inorganic phosphorus as a prognostic test in azotemic nephritis at the time was due to the question which had just been raised as to the accuracy of the creatinine estimation by Benedict.[5] He states: "A plea may therefore be uttered for the value of the plasma content in inorganic phosphorus (phosphate) in estimating the probable outcome of a case of azotemic nephritis." He regarded an inorganic phosphorus of 2 to 4 mg. per 100 c.c. of plasma as normal, and above 10 mg. as a grave prognosis.

To secure information on this point, and on the serum calcium, Schmitz, Rohdenburg and Myers[63] studied a series of 54 cases with markedly impaired renal function. On the basis of their data it would appear that the values for the creatinine and serum inorganic phosphorus are equally significant, but that the fluctuations in the blood urea are so great as to be much less significant. However, it was the authors' opinion on the basis of following the serial findings

in the individual cases that there was little doubt about the reliability of inorganic phosphorus retention as a prognostic sign in chronic nephritis, but that creatinine is equally reliable and the retention appears to occur appreciably earlier than is the case with the inorganic phosphorus.

Diazo-Color Reaction.—Andrewes,[64] while carrying out the van den Bergh reaction for bilirubin on blood serum, noted a deviation from the normal color. When alkali was added to the end-product a pink coloration was obtained instead of the green of azobilirubin. This reaction was found to occur only in persons with markedly damaged kidneys. Hewitt[65] modified the method so as to rule out false positives.

According to Harrison and Broomfield[66] the substance responsible for the reaction is an indoxyl compound, presumably indican, or possibly, in part, indoxyl glycuronate. It is therefore not surprising that the results obtained with the diazo reaction harmonize with the indican test (see below).

Rabinowitch[67] has apparently made a more extensive clinical study of this test than anyone else (also gives literature). He observed that when an attempt was made to correlate the positive reactions with the urea content of the blood no correlation was noted. This, he felt, was to be expected, since the prognosis and degree of retention of blood urea are not related. Although Rabinowitch made many hundreds of analyses, and frequently found high blood ureas associated with negative diazo-color reactions, he never found a positive diazo-color reaction associated with a normal blood urea nitrogen. In the case of creatinine Rabinowitch commented: "here there appears to be a relationship," his observations being reproduced in Table III. He noted two cases with creatinine values greater than 5 mg. per 100 c.c. of blood with negative diazo-color reactions, but in these cases the anurias were due to mechanical block (prostatic hypertrophy). The conclusion was drawn that a positive diazo-color reaction is not found in any condition other than severe kidney damage, although a positive reaction may be found in persons with severe kidney damage, and such persons may recover. Such cases include the albuminurias of severe infections, the well-recognized form

TABLE III

RELATIONSHIP BETWEEN POSITIVE DIAZO-COLOR REACTION AND CREATININE CONTENT OF BLOOD (RABINOWITCH[67])

BLOOD CREATININE	INCIDENCE OF POSITIVE DIAZO-COLOR REACTION
mg. per 100 c.c.	
-1.50	0
1.51-1.75	0
1.76-2.00	0
2.01-2.25	3
2.26-2.50	5
2.51-2.75	5
2.76-3.00	6
3.01-3.25	8
3.26-3.50	7
3.51-3.75	11
3.76-4.00	14
4.01-4.25	16
4.26-4.50	20
4.51-4.75	15
4.76-5.00	26
5.01-	41

of acute nephritis with pallor, edema, etc., acute exacerbations of chronic nephritis, mechanical obstruction in the urinary flow, surgical lesions of the kidneys with urinary retention, and the anuria of diabetic coma. When acute exacerbations of the disease are excluded, a positive reaction occurring in the course of chronic nephritis indicates an unfavorable prognosis.

Indican.—In 1911 Obermayer and Popper[68] called attention to the fact that indican (indoxyl potassium sulfate) is increased in the blood of patients with uremia. Later Rosenberg[69] compared the blood indican and creatinine, as noted above, observing that in azotemia urea was elevated first, then creatinine and finally indican. However, he appeared to favor creatinine as a prognostic test, partly because of the greater simplicity in its estimation. A large literature has accumulated on the subject. Among papers which might be mentioned in chronological order are those by Haas,[70] Monias and Shapiro,[71] Keith and Wakefield,[72] Sharlit,[73] Polayes and Eckert,[74] and Kenny and Hubbard.[75]

The normal for the blood indican is generally given as 0.05 to 0.15 mg. per 100 c.c. of plasma or serum. Values of 0.2 mg. are regarded as abnormal but the values may reach 10 mg. per 100 c.c. in severe uremia. Sharlitt[73] has described a new method which possesses several desirable features. He regards the normal as less than 0.06 mg. and found values to range from 1.55 to 6.20 mg. in ten pathological cases.

Monias and Shapiro[71] have presented data on over a hundred cases in which urea and creatinine were also determined, and regard the indican as definitely superior to creatinine and urea as a prognostic test in uremia. A study of their data, however, reveals the fact that in the cases in which the urea and creatinine findings were less significant than the indican, the blood analyses were made a month or more before the death of the patient. Keith and Wakefield[72] regard an elevation of the indican of the blood as somewhat comparable to the elevation of creatinine, and therefore of prognostic and not of diagnostic significance. While indican is increased by intestinal obstruction, chronic suppuration or infections of long standing and hepatic insufficiency, its elevation in the blood is most marked in renal insufficiency. To visualize the comparative significance of data for urea, creatinine, indican and the xanthoproteic reaction, observations made by Kenny and Hubbard are presented in Table IV. The Table includes only cases in which creatinine determinations were made, the data being rearranged according to the magnitude of the creatinine. On the basis of the data on these 13 cases, the findings for creatinine, indican and the xanthoproteic readings harmonize very well. However, in this series, the creatinine findings appear superior to the indican and about equal to the xanthoproteic readings.

Xanthoproteic Reaction.—The xanthoproteic reaction on serum filtrate as a measure of renal insufficiency was introduced by Becher[76] in 1924. His observation that the test constitutes a reliable prognostic index in uremia has been confirmed by a number of workers, among whom may be mentioned Rasmussen,[77] Steen,[78] Kenny and Hubbard,[75] and recently Ragins, Kraus and Coe.[79] The test is a simple one, consisting essentially of carrying out the xanthoproteic reaction on the trichloroacetic acid filtrate of a measured amount of serum and matching the color against a standard bichromate solution.

TABLE IV

CREATININE, UREA, AND INDICAN AND XANTHOPROTEIC TESTS IN RENAL INSUFFICIENCY REARRANGED ON THE BASIS OF THE MAGNITUDE OF THE CREATININE (DATA OF KENNY AND HUBBARD[75])

CASE	CREATININE	UREA N	INDICAN	XANTHOPROTEIC READING	DIAGNOSIS, REMARKS
16	31.6	203	2.9	144	Malignant nephrosclerosis. Nausea, vomiting, died, uremia, no autopsy.
3	20.3	193	2.0	142	Chronic glomerulonephritis. Drowsy, muscle twitching, died, uremia, no autopsy.
18	14.1	244	0.8	153	Chronic glomerulonephritis. Nausea, vomiting, coma, died, uremia, no autopsy.
9	13.0	123	3.5	83	Comatose, uremic frost. Autopsy: Malignant nephrosclerosis, uremia.
2	12.8	148	Tr.	133	Comatose. Autopsy: Carcinoma of cervix, pyelonephritis, uremia.
10	9.0	134	3.0	100	Comatose. Autopsy: Hypertrophied prostate, hydronephrosis, uremia.
1	9.0	106	1.7	90	Pyelonephritis, uremia, died, no autopsy.
5	8.2	95	2.1	111	Tubercular kidney, uremia. Nausea, vomiting, death, no autopsy.
8	5.4	64	2.5	100	Chronic glomerulonephritis. Vomiting, died at home.
4	5.3	109	2.3	181	Vomiting. Autopsy: Combined arteriosclerosis and nephritis.
2	4.5	88	Tr.	50	Hypertrophied prostate, pyelonephritis. Operation and improvement.
3	1.3	40	Neg.	62	Cystotomy, death not due to renal insufficiency.
20	1.2	12	Neg.	15	Convulsions. Death: Acute glomerulonephritis, lobar pneumonia.

The normal range of values has generally been given as 25 to 35, but Ragins et al. believe that a more correct normal range is 0 to 50. They consider that xanthoprotein values over 60 should be considered abnormal in all conditions.

Becher[80] further reported that in the blood of patients with renal insufficiency there was an increase of aromatic substances—an ether soluble group comprising phenols, cresols, aromatic oxyacids and indoxyl, and a non-ether group containing the amino acids, tyrosine, phenylalanine and tryptophane—and have associated this with the increased xanthoproteic reaction. Later with Koch[81] he reported markedly increased xanthoproteic values (80 to 250) in patients with uremia and indicated that uremia was less dependent on retention of nitrogenous products than on that of aromatic substances, and that phenol poisoning resembled true uremia. Kenny and Hubbard[75] feel that the xanthoproteic reaction is of special value in distinguishing between true and pseudo-uremia. They state that in pseudo-uremia, or hypertensive encephalopathy, the symptoms are due to cerebral edema and the xanthoproteic reaction remains low regardless of the urea nitrogen. Ragins et al.[79] concluded from their data that uremia was the only specific condition giving a significantly increased xanthoproteic reaction, although lysol poisoning must be kept in mind as a possibility. A few illustrative observations from the data of Kenny and Hubbard are given in Table IV and show the comparative value of the test.

RESPONSE TO INTRAVENOUSLY ADMINISTERED CREATININE

In 1923 Major[82] suggested following the two-hour output of creatinine after the intravenous administration of 0.5 Gm. as a test of renal function. He observed that the normal kidneys responded to the intravenous injection of creatinine by an increased output during the same period of time, while in chronic nephritis the kidneys showed on the average only a slight increase, and in many instances a decrease. This test never came into general use for the probable reason that it required the intravenous administration of an expensive compound in sterile solution.

CREATININE CLEARANCE TEST

The creatinine clearance test introduced by Rehberg has been studied by a number of workers as a test of renal function. Hayman, Halsted and Seyler[83] made a careful comparison of this test with the urea clearance test to determine if there was sufficient practical advantage in the creatinine test to compensate for the added technical difficulties. In close agreement with Möller, McIntosh and Van Slyke, they found in a series of about 25 normal individuals that the maximum urea clearance averaged 75 c.c. per minute, while the mean standard urea clearance was 51 c.c. per minute. The mean creatinine clearance on about 60 normal subjects was 148 c.c. per minute. This would appear to indicate that the creatinine is normally eliminated two or three times as readily as the urea. However, Hayman et al. found that in patients with Bright's disease the creatinine and urea clearance tests were generally equally reduced in relation to the average normal. Since the variability of the two tests from the average normal was approximately the same, they concluded that the creatinine clearance test did not possess any practical advantages to compensate for its greater technical difficulty (ingestion of 3 to 5 Gm. of expensive creatinine and the analysis of two blood samples).

DISCUSSION

On the basis of the tests discussed it would appear that the Van Slyke urea clearance test gives the most reliable measure of renal insufficiency, especially in the early stages of renal disease. When definite impairment in renal function has been established the level of the blood urea nitrogen should also be taken into consideration, since this is available from the data required for the calculation of the urea clearance. With a definite elevation of the blood urea nitrogen, the creatinine should also be determined. The author believes that practical experience has conclusively demonstrated the superiority of the blood creatinine over the urea nitrogen as a prognostic sign in cases with markedly impaired renal function. While essentially the same prognosis might be given in many cases on the basis of figures for the urea nitrogen alone, there are a considerable number where the creatinine may be elevated above the critical level without the presence of correspondingly high values for the urea nitrogen. It is on this account that the prognostic significance of an elevated blood creatinine is more dependable than that of urea. Theoretically this finding would seem logical since creatinine is more readily eliminated than urea, is excreted by the human kidney somewhat differently than urea in that the tubules participate, and

furthermore is almost entirely endogenous whereas urea under ordinary conditions of diet is largely exogenous in origin. The statement may be made that the urea clearance test is also a good prognostic test. This is true but with marked retention the changes in the blood creatinine are greater and therefore become more significant. The blood creatinine furthermore is an excellent confirmatory test in cases in which low values for the urea clearance are found. It should also be borne in mind that the urea clearance test requires an accurate urine collection, which is sometimes impossible. The creatinine on the other hand requires simply an analysis of the blood, but it would be well in this case always to include determination of urea as well as creatinine.

While the phenolsulfonphthalein test has proved itself an extremely valuable renal function test, the output of the dye is so low with severe renal impairment as not to adequately reflect the changes at this stage in the disease.

The rise in the level of the inorganic phosphorus in advanced renal disease is an infallible sign of marked impairment in renal function. In the author's opinion, however, it comes a little later than the retention of creatinine. On this account the creatinine determination would appear superior as a prognostic test.

The value of the determination of indican, the diazo reaction and the xanthoproteic reaction have all been amply demonstrated. Many of the investigators who have worked with these tests have also presented parallel figures for the blood creatinine and urea. While the reliability of these special tests has been proved, in the author's opinion these tests have not been shown to have any greater reliability than the blood creatinine. On this account the estimation of the blood creatinine would appear to remain the method of choice on account of greater simplicity as a routine chemical blood determination.

SUMMARY

The prognostic significance of an elevated creatinine has been discussed on the basis of individual observations and those in the literature. The test has also been considered in comparison with the following tests which have also been advocated for the same purpose, viz., the phenolsulfonphthalein test, the blood urea, the Van Slyke urea clearance test and the serum inorganic phosphorus, indican, diazo-color reaction, and xanthoproteic reaction, also the response to intravenously administered creatinine and the creatinine clearance test.

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CLINICAL AND EXPERIMENTAL

HEMATOLOGIC COMPLICATIONS OF THERAPY WITH RADIOACTIVE PHOSPHORUS

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DURING the past two and one-half years, 116 patients with various types of hematologic dyscrasias have been treated at the Washington University School of Medicine with radioactive phosphorus. Of these, 100 have been observed for a sufficiently long period of time to permit evaluation of the therapy. The results in general give weight to the conclusions of other investigators that radioactive phosphorus is a valuable therapeutic agent in the management of polycythemia vera and the chronic forms of leukemia.[1-11] Effectiveness in patients with most acute leukemias, Hodgkin's disease, reticulum cell sarcoma, and lymphosarcoma have been disappointing. A detailed analysis of these therapeutic results will be made elsewhere. The primary purpose of this communication is to call attention to the frequency with which anemia, leucopenia, and thrombocytopenia developed in the patients under treatment. In most instances, there was excellent evidence that these complications were induced by the therapy and were not manifestations of the disease per se. They frequently appeared weeks or several months after the last injection of radioactive phosphorus had been given. In the patients with polycythemia, they were often produced by amounts of radioactivity significantly less than those used in other clinics.[10, 11]

Since it is well known that excessive exposure to radium and x-ray causes toxic effects on the bone marrow, these hematologic complications are not unexpected. With x-ray and radium, however, at least when used in therapeutic doses, the changes induced in the peripheral blood appear promptly.[12, 13] As indicated above, this is frequently not true for radioactive phosphorus, presumably because with its half-life of 14.3 days it continuously emits radiant energy for weeks after administration. It is not surprising, therefore, that the appearance of toxic effects may be delayed for many weeks. While no emphasis has previously been given to this possibility, Scott and Cook[14] and Scott and Lawrence[15] have studied the effect of radioactive phosphorus on the blood of growing chicks and of monkeys. In chicks, with the dosages used, the polymorphonuclear leucocytes became greatly reduced in numbers, but there were no significant changes in the other formed elements. In monkeys, radiophosphorus lowered the absolute numbers of lymphocytes, granulocytes, and

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The expenses of this study were defrayed in part by a grant from the Commonwealth Fund.

Presented in part before the 16th Annual Meeting of the Central Society for Clinical Research, Chicago, Nov. 5, 1943.

Received for publication, Aug. 2, 1944.

erythrocytes. The changes occurred chronologically in the order as listed. One of the animals died following a dosage of 1.04 millicuries per pound of body weight; its bone marrow was aplastic. Scott and Lawrence emphasize, however, that the tolerance levels were about ten times those used therapeutically. Several of the reports describing clinical experience with the use of radioactive phosphorus contain examples of toxic effects on the bone marrow. Kenney and Craver mention one patient with lymphosarcoma who developed an aplastic anemia under treatment, one who developed a temporary anemia, and several who had transient leucopenia.[16] Erf, Tuttle, and Lawrence[5] show that several of their patients with lymphatic leukemia who were treated with radioactive phosphorus showed significant lowering of the platelet count while they were apparently improving otherwise; in no instances were hemorrhagic manifestations reported. In one of eleven patients with polycythemia, Erf and Jones noted a fall in platelets from 180,000 to 64,000 per cubic millimeter.[11] Kenney reported that in one of his patients with acute lymphatic leukemia the terminal appearance of the bone marrow was one of extreme aplasia with multiple hemorrhages.[7] He stated that: "The marrow changes were more severe than those ever seen in this institution (Memorial Hospital, New York City) in terminal leukemia not treated with radioactive phosphorus." Diamond and Warren, furthermore, observed that several children with acute leukemia whom they treated with radioactive phosphorus had aplastic marrows at autopsy.[17]

The diagnoses established for the 100 patients included in this study were polycythemia vera, 18; chronic myelogenous leukemia, 25; chronic lymphatic leukemia, 22; leucosarcoma, 13;[18] monocytic leukemia, 4; plasma cell myeloma, 4; lymphosarcoma, 2; reticulum cell sarcoma, 3; Hodgkin's disease, 6; giant follicular lymphoblastoma, 1. Patients were accepted for treatment irrespective of the stage of their disease or the previous treatment they had received. They were refused only when their residences were so far from St. Louis as to preclude frequent visits to the clinic.

MATERIALS AND METHODS

The radioactive phosphorus was prepared in the medical cyclotron of the Mallinckrodt Institute* by bombardment of red phosphorus by 12 million volt deuterons. The phosphorus was then synthesized into its dibasic sodium salt, according to the procedure used by Kamen,[19] and was dissolved in enough water to make an isotonic solution (15 mg. per cubic centimeter).† The radio-

*Built with funds provided by the Rockefeller Foundation.

†The details of the method are as follows:

1. The phosphorus from the target is washed first with water, then with aqua regia.
2. 100 c.c. aqua regia are added and the mixture is boiled until syrupy to oxidize the P to P_2O_5.
3. Small amounts of conc. HCL are added and heating is continued until no more brown fumes are given off (indicating that nitric acid has gone). There is no danger of evaporating off the phosphorus pentoxide as there is enough copper present to form copper phosphate.
4. The digest is cooled and diluted with water to about 200 c.c.
5. H_2S is bubbled through the solution for from one to two minutes to precipitate the copper. CuS suspension will coagulate with heat if there is no nitric acid present. The liquid is freed of precipitate by filtration through a Buechner funnel.
6. The filtrate is evaporated carefully so as not to lose the P_2O_5. As long as the liquid continues to fizz, HCL is present; when white fumes appear, the evaporation has been carried too far. The phosphorus is now in the form of H_3PO_4.
7. It is neutralized to phenol red with 1N NaOH.
8. Any copper precipitated as copper hydroxide is removed by filtration and the solution is diluted to isotonic concentration. The amount of phosphorus present can be calculated from the amount of NaOH required to neutralize the phosphoric acid. Actually 4.85 c.c. of water are required in the final dilution per 1 c.c. 1N NaOH used in the titration. The isotonic solution of Na_2HPO_4 contains 0.15 mg. of the salt per cubic centimeter.

activity of the phosphate solution was determined by means of a Lauritson electroscope calibrated with a uranium standard. The initial activity of a freshly prepared solution usually varied between 300 and 400 microcuries per cubic centimeter. The solution of radioactive phosphorus was used during a period of weeks until its strength had decayed to 30 to 50 microcuries per cubic centimeter.

The plan of treatment for all the diseases except polycythemia closely followed the "fractional method" of therapy outlined by Low-Beer, Lawrence, and Stone.[19] This refers to small frequent intravenous injections of radioactive phosphorus. In only a very few instances was the material given by mouth. The size of each dose given parenterally varied between 100 and 2,000 microcuries. Patients were usually treated two or three times a week at first. As the white blood cell count either approached normal in the patients with leukemia or became subnormal in the cases where the white blood count was not elevated at the onset of treatment, the dosage was decreased and the time interval between treatments was lengthened. Patients with polycythemia were given larger quantities of radioactive phosphorus at much less frequent intervals.

Therapy was governed principally by the changes in the peripheral blood. An attempt was made in each case to restore the cytology of the blood to as near normal as possible and to maintain this effect. Considerable individualization of treatment was necessary because of the great variation in the response shown by different persons. The remissions induced in most patients were sufficiently good to permit interruption of therapy for a period of months. In a few instances it seemed desirable to continue regular injections of the radioactive phosphorus at intervals of several weeks. More active therapy was reinstituted whenever the changes in the peripheral blood indicated that the remission had ended, even though no symptoms developed at these times.

In the cases of lymphosarcoma, Hodgkin's disease, reticulum cell sarcoma, multiple myeloma, and giant follicular lymphoblastoma, where the white blood cell count was not elevated, regulation of therapy was more difficult. Administration of radioactive phosphorus was continued in these instances until changes in the peripheral blood gave warning of a depression of bone marrow activity. In some patients serial bone marrow aspirations were done to aid in regulating therapy. Patients with subleukemic leukemia were given amounts that were comparable with those required to regulate the subjects with elevated counts.

On each visit to the clinic, the patient was examined and the following laboratory data were obtained: total erythrocyte and white blood cell counts, hemoglobin, reticulocyte level, platelet count, and a blood film examination. While fixed cover slip preparations were made for permanent records, the differentials were made routinely with the supravital technique. The blood-counting equipment had all been standardized by the U. S. Bureau of Standards. Hemoglobin determinations were made by the Evelyn oxyhemoglobin method[20] on an Evelyn photometer that had been standardized by the simultaneous determinations of the oxygen-combining power. Reticulocyte and platelet counts were done with the Dameshek method.[21]

Diagnoses were established by study of the peripheral blood and sternal marrow removed by aspiration and by biopsy of lymph nodes and bone marrow. All eighteen patients with polycythemia vera had palpable spleens and were shown to have an increase in the total circulating blood volume. In addition, clinical study proved the absence of pulmonary or circulatory factors which could cause secondary polycythemia. In a few instances the degree of saturation of arterial blood with oxygen was measured.

RESULTS

The incidence, among these patients, of thrombocytopenia, leucopenia, and anemia is summarized in Table I. Sometimes two or all three changes were observed in the same individual. If so, the pattern most frequently seen was first a fall in the white count, then in the platelets, and lastly in the erythrocyte level. It should be emphasized, however, that the platelets occasionally fell to very low levels even in patients who did not develop a leucopenia or an anemia.

A. *Thrombocytopenia.*—Perhaps the most striking complication was the thrombocytopenia. The platelet count fell to below 100,000 per cubic millimeter in forty-four instances and to below 50,000 in thirty-three of these. These figures do not include any patients in whom the fall in platelets was obviously a manifestation of the dyscrasia. It frequently developed, in patients with leukemia, for instance, several weeks or months after the white cell count had approached normal and most of the abnormal leucocytes had disappeared from the peripheral blood. In some cases, the thrombocytopenia occurred even after therapy had been discontinued. An excellent example is provided by the case of J. M., a man with chronic myelogenous leukemia (Fig. 1). The remission induced in him was the best ever obtained in this series of cases. He received only 10.073 millicuries of radioactive phosphorus in a period of fifty-two days, during which time his white cell count fell from 111,000 to 10,500 cells per cubic millimeter. There was striking clinical improvement with an attendant decrease in the size of his spleen. Yet four weeks after the last injection of radioactive phosphorus and six weeks after the white cell count had returned to a normal range and myelocytes had almost entirely disappeared from the differential, the platelets began to fall. When they reached 40,000 per cubic millimeter, it was necessary to hospitalize the patient because he was bleeding from his gums and complained of overwhelming weakness. There was no fever. Two transfusions were given, and the platelets slowly returned to normal levels. The thrombocytopenia in this instance could hardly be related to an exacerbation of the disease.

Thrombocytopenia is extremely rare in polycythemia vera. For that reason, its occurrence in N. W., a 59-year-old woman with polycythemia vera, after she had received only 7.56 millicuries of radioactive phosphorus, is particularly interesting (Fig. 2). The 7.56 millicuries was all given orally in two doses on succeeding days. This was one of the very few cases in which the phosphorus solution was given by mouth; and, if it is assumed that 75 per cent was absorbed from the gastrointestinal tract,[22] this amount would be equivalent to 5.67 millicuries by intravenous injection. This woman returned to the hospital before her scheduled appointment because of moderately ex-

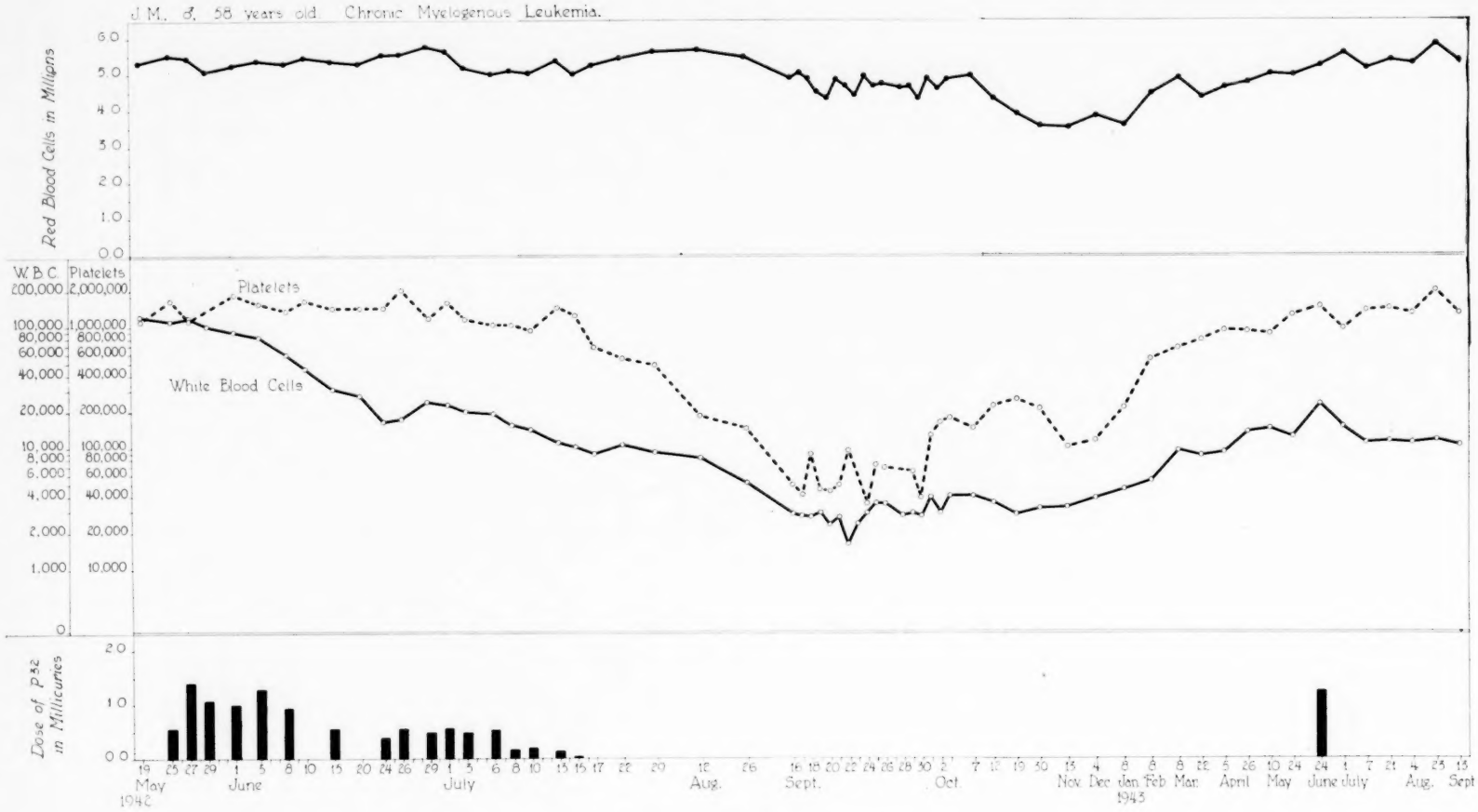

Fig. 1.—For legend see opposite page.

FIG. 1

THROMBOCYTOPENIA AND LEUCOPENIA IN A PATIENT WITH CHRONIC MYELOGENOUS LEUKEMIA FOLLOWING THERAPY WITH P^{32}

DIFFERENTIAL COUNTS

			MYELOCYTES							
DATE	EOSIN.	BASO.	A	B	C	JUVENILES	STAB	SEG.	L.	M.
5 25/42	0	9	0	2	22	22	13	26	2	4
6/ 8/42	1	12	0	2	4	22	21	34	2	2
7/ 6/42	0	5	0	0	6	7	6	60	9	7
8/12/42	1	2	0	0	1	0	1	73	14	8
9/22/42	**2**	**0**	**0**	**0**	**0**	**0**	8	38	44	8
2/ 8/43	1	5	0	0	2	3	3	57	10	19
9/13/43	1	5	0	0	2	1	2	74	9	6
5/31/44	1	9	0	0	1	2	5	67	12	3

From Sept. 13, 1943, the last date included on the above chart, to May 31, 1944, this patient's blood counts have all remained essentially normal, except for a rise of the leucocyte count to 19,450 in May, 1944. The white count promptly returned to normal after two injections of radioactive phosphorus (1.136 millicuries and 0.518 millicuries, respectively).

Case Summary.—J. M., a 58-year-old married man, first noted slight fatigability sometime during 1940. In January, 1941, the leucocyte count was found to be 88,000 and the diagnosis of myelogenous leukemia was established. He was given x-ray irradiation totaling 750 roentgen units, whereupon the white cell count dropped to 12,000. He felt very well and was entirely asymptomatic. However, the leucocyte count gradually rose, and on May 25, 1942, radioactive phosphorus therapy was started because the white cell count had reached 111,000.

Physical Examination (May 25, 1942).—Blood pressure was 155/100. The skin was clear. Lymph nodes were not palpably enlarged. The liver edge was felt 2 cm. below the right costal margin, and the spleen was felt 2 cm. below the left costal margin.

Laboratory Data (May 25, 1942).—Urine was normal. Kahn test was negative. White blood cells, 111,000; red blood cells, 5,510,000; hemoglobin, 15 Gm.; reticulocytes, 2.6 per cent; platelets, 1,664,000; differential: basophiles, 9 per cent; "B" myelocytes, 2 per cent; "C" myelocytes, 22 per cent; juveniles, 22 per cent; stabs, 13 per cent; segmented neutrophiles, 26 per cent; lymphocytes, 2 per cent; monocytes, 4 per cent.

A bone marrow examination done a year previously (May 15, 1941) showed a hyperplastic marrow in which 51 per cent of the cells were myelocytes. Very few nucleated red blood cells were seen.

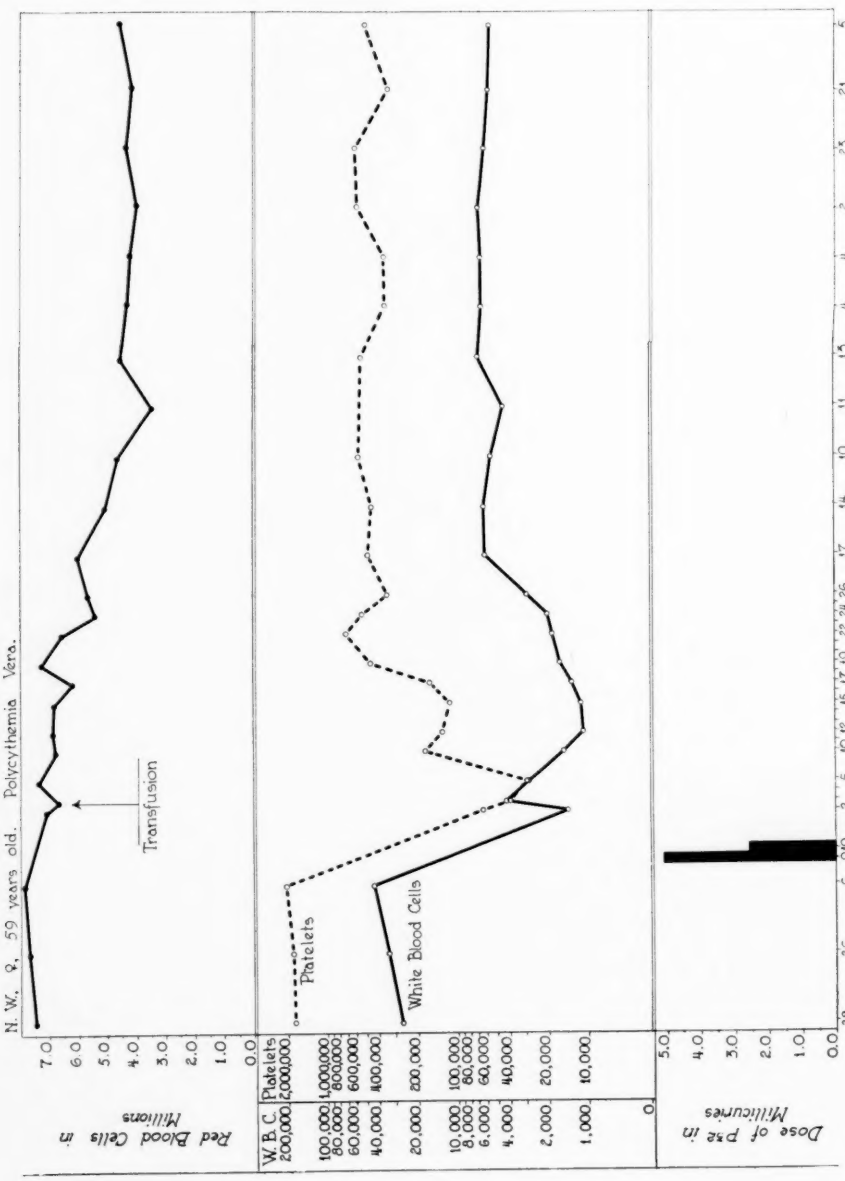

Fig. 2.—For legend see opposite page.

FIG. 2

THERAPEUTICALLY INDUCED LEUCOPENIA AND THROMBOCYTOPENIA IN A PATIENT WITH POLYCYTHEMIA VERA

Case Summary.—N. W., a 59-year-old married woman, had always enjoyed good health until the summer of 1940 when she began to tire easily. At about the same time she noted that the fingers were purplish red in color. Weakness increased and gradually the skin of the entire body became bluish red. In the spring of 1941 she became aware of a mass in the right upper quadrant of the abdomen; there was an occasional aching pain in this region. During the following year she developed sharp pains in her finger tips, aching of her legs, pounding headaches, vertigo and tinnitus, dyspnea on exertion, depression, forgetfulness, nosebleeds, and visual disturbances.

The patient was admitted to Barnes Hospital in February, 1942. The erythrocyte count at that time was 10,535,000 per cubic millimeter and the hemoglobin was 22.5 Gm. Treatment consisted of repeated venesections; a total of 4,200 c.c. of blood was removed. At the time of discharge from the hospital the erythrocyte level was 5,800,000 per cubic millimeter, and the hemoglobin was 14.7 Gm. Symptomatically, she was considerably improved.

For the following year she was treated by periodic venesection, averaging one pint of blood withdrawn every eight weeks. After each phlebotomy she felt weak for a day, following which she would be relatively asymptomatic for from four to six weeks. There would then be a gradual return of all her symptoms.

Radioactive phosphorus therapy was given Jan. 9 and 10, 1943.

Physical Examination (Jan. 9, 1943).—Blood pressure was 170/110. The finger tips, lips, ears, tongue, and pharynx were purplish red. There were numerous small petechiae over the shoulders and lower extremities and a large bruise on the left forearm. Lymph nodes were not palpable. The heart was moderately enlarged, and a systolic murmur was heard at both the apex and base. The lungs were clear. A firm, nontender spleen was felt extending 4 cm. below the left costal margin; the liver edge was felt 5 cm. below the right costal margin.

Laboratory Data.—Urine was normal. Kahn test was negative. Basal metabolic rate, +20 per cent. Blood counts (Jan. 6, 1943): red blood cells, 7,830,000; hemoglobin, 12.5 Gm.; white blood cells, 43,700; reticulocytes, 1.6 per cent; platelets, 2,040,000. The differential was normal. The bone marrow was examined but not until a week after radioactive phosphorus therapy had been given; the marrow was hyperplastic but the differential was not unusual except for the presence of large numbers of primitive cells.

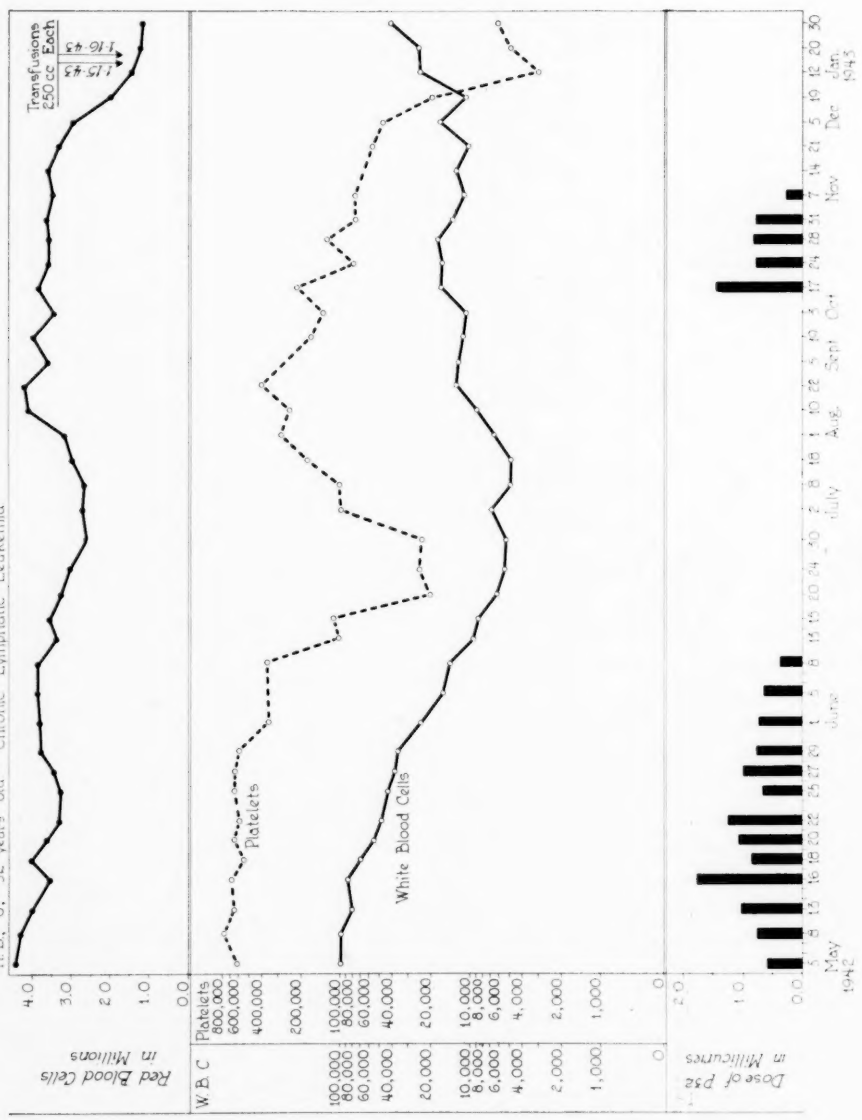

Fig. 3.—For legend see opposite page.

FIG. 3

THROMBOCYTOPENIA OCCURRING IN A PATIENT WITH CHRONIC LYMPHATIC LEUKEMIA FOLLOWING THERAPY WITH P^{32}

Case Summary.—H. B., a 52-year-old married man, first noticed a lump in the left side of the neck in January, 1938. Shortly thereafter the blood was examined and he was told he had leukemia. He was treated with capsules (contents unknown). Very gradually over a period of several years other glands became enlarged.

There were no constitutional symptoms until October, 1941, when the nodes became so large as to make it difficult for him to swallow or turn his neck. He felt weak and tired, and his gums bled whenever he brushed his teeth. He was given ten x-ray treatments totaling 1,000 roentgen units. There was transient symptomatic improvement with decrease in the size of his glands, but six weeks later his glands were larger than before. In January, 1942, he was given x-ray therapy again (nine treatments, totaling 790 roentgen units). This time improvement lasted only about a month. From February to May, 1942, the glands increased in size, he developed pain in his back, and became progressively weaker. Treatment with radioactive phosphorus was started May 5, 1942.

Physical examination at that time revealed enlarged lymph nodes in the neck, axillae, and inguinal regions; some of these were large enough to have a diameter of 4 cm. Even enlarged iliac glands were easily felt. There was slight edema of the face and neck. The edge of the spleen was felt 5 cm. below the costal margin. There were no other significant findings.

Laboratory Data (May 5, 1942).—White blood cells, 98,500; red blood cells, 4,410,000; hemoglobin, 12.8 Gm.; reticulocytes, 0.8 per cent; platelets, 620,000; differential: myelocytes, 1 per cent; stabs, 1 per cent; segmented neutrophiles, 8 per cent; lymphocytes, 89 per cent; monocytes, 1 per cent. A lymph node was biopsied Aug. 8, 1942; the microscopic appearance of this gland was compatible with the diagnosis of lymphatic leukemia.

The patient died June 12, 1943; during the last four and one-half months of life, he was a patient at another hospital. The erythrocyte and thrombocyte levels remained very low and the leucocyte level fluctuated widely.

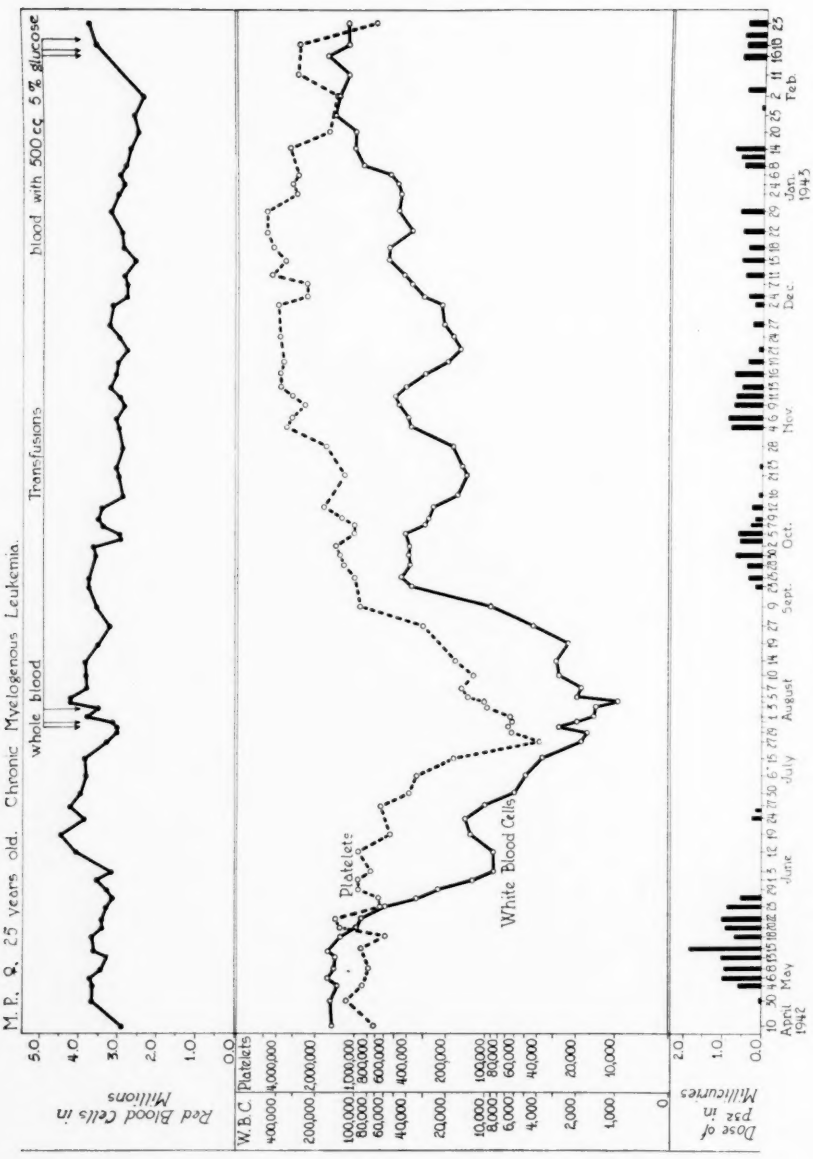

Fig. 4.—For legend see opposite page.

FIG. 4

THROMBOCYTOPENIA AND LEUCOPENIA IN A PATIENT WITH CHRONIC MYELOGENOUS LEUKEMIA FOLLOWING THERAPY WITH P^{32}

Case Summary.—M. P., a 25-year-old married woman, developed an itchy, scaly eruption on both eyebrows in March, 1939. This eruption spread down over the lids, and finally over and behind the ears. X-ray treatments and salves were without benefit.

In June, 1939, she had a miscarriage and routine blood counts at this time were: white blood cells, 14,000; red blood cells, 4,470,000; hemoglobin, 87 per cent; differential: segmented neutrophiles, 66 per cent; stabs, 7 per cent; lymphocytes 27 per cent. She was again hospitalized in June, 1941, when she delivered a normal infant. Blood counts at this time were: white blood cells, 28,100; red blood cells, 4,230,000; hemoglobin, 81 per cent; differential: segmented neutrophiles, 84 per cent; stabs, 7 per cent; lymphocytes, 9 per cent.

Aside from the eruption, which persisted, the patient had no symptoms until January, 1942, when she developed weakness and fatigue. These symptoms persisted until April 30, 1942, when treatment with radioactive phosphorus was started.

Physical Examination (April 30, 1942).—There was a symmetrical skin eruption involving the eyelids, brows, ears, and adjacent areas; the lesion was slightly pigmented, thickened, dry, and scaly, with slightly raised borders. The lymph nodes were not palpably enlarged. The liver and spleen were not palpable (both liver and spleen edges were palpated 2 cm. below the costal margin on May 30, 1942). There were no other significant findings.

Laboratory Data.—Urine was normal. Kahn test was negative. Blood counts (April 30, 1942): white blood counts, 155,000; red blood cells, 3,690,000; hemoglobin, 10.8 Gm.; reticulocytes, 3 per cent; platelets, 1,180,000; differential: basophiles, 7 per cent; eosinophiles, 2 per cent; myeloblasts, 2 per cent; "B" myelocytes, 6%; "C" myelocytes, 25 per cent; juveniles, 18 per cent; stabs, 19 per cent; segmented neutrophiles, 17 per cent; lymphocytes, 1 per cent; monocytes, 3 per cent. A bone marrow examination was not done until Aug. 18, 1942, 106 days after treatment was started. The bone marrow at this time showed myeloid hyperplasia (52 per cent myelocytes) and only 14 nucleated red blood cells per 100 white blood cells.

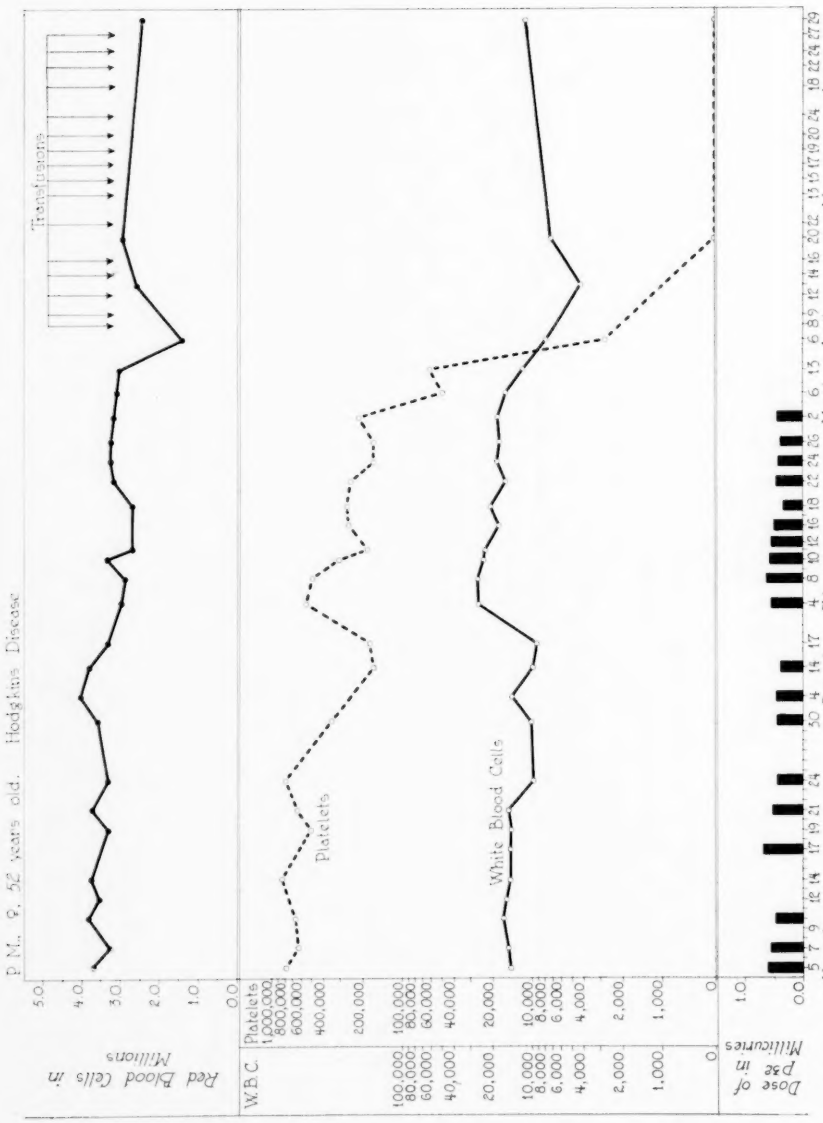

Fig. 5.—For legend see opposite page.

FIG. 5

THROMBOCYTOPENIA FOLLOWING TREATMENT WITH P^{32} IN A PATIENT WITH HODGKIN'S DISEASE

Case Summary.—P. M., a 52-year-old married woman, enjoyed excellent health until August, 1941, when she first noted a dry hacking cough. In March, 1942, the cough became productive of mucoid sputum. About this same time she began to have dull, aching pain in the left lower chest posteriorly. Shortly thereafter she began to have severe night sweats and increasing fatigue. She lost 12 pounds in weight. An x-ray of the chest in June, 1942, showed extensive pulmonary infiltration. Bronchoscopy revealed only chronic inflammation of the bronchi. By August, 1942, enlargement of the axillary and inguinal lymph nodes was apparent; a biopsy (right axillary node) revealed changes typical of Hodgkin's disease. She was given nine x-ray treatments to the right chest and axilla, following which the pain and cough disappeared. She gained nine pounds. During October the nodes in the left groin increased steadily in size and became quite sore and tender. Radioactive phosphorus therapy was started Nov. 5, 1942.

Physical Examination (Nov. 5, 1942).—There was x-ray pigmentation over the right chest and back. A large matted mass of lymph nodes measuring 4 by 3 by 3 cm. was felt in the left inguinal region. The heart and lungs were normal. A firm mass thought to be spleen was felt in the left upper quadrant of the abdomen, but a distinct edge could not be outlined.

Laboratory Data.—Urine was negative except for a faint tract of albumin. Kahn test was negative. Blood counts (Nov. 5, 1942): white blood cells, 14,700; red blood cells, 3,690,000; hemoglobin, 10.1 Gm.; reticulocytes, 2.8 per cent; platelets, 790,000; differential: eosinophiles, 25 per cent; juveniles, 1 per cent; stabs, 4 per cent, segmented polymorphonuclear neutrophiles, 48 per cent; lymphocytes, 16 per cent; and monocytes, 6 per cent. Sternal bone marrow examination showed 43 per cent eosinophiles, 39 per cent lymphocytes, 6 per cent plasma cells, and only 12 per cent cells of the myeloid series. Very few nucleated red blood cells were seen. There were many globules of fat present.

An x-ray of the chest showed considerable increase in the amount of infiltration in each lung.

tensive purpura on the lower extremities. The platelet count had fallen from 2,000,000 per cubic millimeter to a level of 29,000. Data illustrating three additional instances of the same change are summarized in Figs. 3 to 5.

Of the 44 patients whose platelet levels dropped to less than 100,000 per cubic millimeter, 38 showed clinical manifestations of abnormal bleeding: 22 developed petechiae, purpura, or ecchymoses; 12 bled from the gums; 10 had epistaxis; 6 had hematemesis, hemoptysis, or bloody stools; and 2 had hematuria. Twelve subjects, not included in the foregoing figures, had a thrombocytopenia before treatment was started. Most of these latter patients had acute leukemia, and all had bleeding manifestations. In no case was it felt that bleeding associated with a thrombocytopenia was the principal cause of death, but in several patients in whom the thrombocytopenia was persistent, the bleeding was profuse and may have contributed to the fatal outcome.

It should be emphasized that in many cases radioactive phosphorus therapy was followed by a significant rise in the platelet level. The cases in which the platelet count rose from definitely thrombocytopenic levels to normal values are summarized in Table II.

Many other patients who did not have an initial thrombocytopenia developed a marked increase in the platelet level after they were treated. In 23 of our 100 patients the platelet count increased by 500,000 or more per cubic millimeter at some time during therapy or shortly after treatment was discontinued.

B. Leucopenia.—Following the institution of radioactive phosphorus therapy, the leucocyte levels followed various patterns. In 56 patients there was a steady, progressive decrease in the leucocyte count, in 25 the white blood cell level first increased and then showed a progressive decrease; in 2 patients with acute leukemia there was a gradual rise in the number of leucocytes up to the time of death. Three patients who were in the terminal stages of leukemia first showed a decrease followed by a progressive increase. In the other 14 there was either no significant change in the leucocyte count or a pre-existing trend was apparently uninfluenced by therapy. Thus treatment was attended by an ultimate decrease in the white blood cell level in 81 of the patients, no significant change in 14, and an ultimate increase in 5 with acute or terminal leukemia. The changes in these last 5 patients were probably manifestations of leukemia; it is impossible to tell whether the radioactivity influenced them in any way.

Further analysis of the 18 cases in which the leucocyte level first increased and later progressively decreased is of interest. When treatment was started, the routine followed was to obtain counts every one to four days for the first two weeks, after which the interval was lengthened to once a week. If daily blood counts had been obtained, more patients might have fallen in this group. The interval between the first injection of radioactive phosphorus and the highest peak of the leucocyte curve varied from one to nine days, with an average of four days. The average increase was 17,000 cells with minimum and maximum values of 1,600 and 86,000, respectively; the pretherapeutic basal values had been fairly constant in each case. These observations have added interest in view of the observations of Isaacs and many others, that x-ray therapy is usually followed by a rise in the leucocyte count before the progressive fall sets in.[23]

TABLE I

THE INCIDENCE WITH WHICH ANEMIA, LEUCOPENIA, AND THROMBOCYTOPENIA OCCURRED IN 100 PATIENTS AS A COMPLICATION OF THERAPY WITH RADIOACTIVE PHOSPHORUS

		ERYTHROCYTES		LEUCOCYTES		PLATELETS		
						THROMBOCYTOPENIA		
	TOTAL CASES	DECREASED 1,000,000 OR MORE (TO < 3.5 M PER C.MM.)	INCREASED 1,000,000 OR MORE PER C.MM.	LEUCOPENIA (FALL TO LESS THAN 3,000 PER C.MM.)	INITIAL COUNT < 2000 ROSE TO > 6,000 PER C.MM.	FALL TO ≤ 100,000 BUT > 50,000 PER C.MM.	FALL TO < 50,000 PER C.MM.	INCREASED BY MORE THAN 500,000 PER C.MM.
Polycythemia vera	18	4	0	9	0	2	1	3
Myelogenous leukemia	25	8	10	7	0	1	10	13
Lymphatic leukemia	22	7	0	5	0	6	6	2
Leucosarcoma	13	9	1	8	2	1	9	2
Monocytic leukemia	4	0	0	0	0	0	0	0
Plasma cell myeloma	6	2	0	4	0	0	3	1
Lymphosarcoma	2	0	0	1	0	0	0	1
Reticulum cell sarcoma	3	2	0	3	0	0	2	0
Hodgkin's disease	6	3	0	3	0	0	2	0
Giant follicular lymphoblastoma	1	1	0	1	0	0	0	1
Total	100	36	11	41	2	10	33	23

TABLE II

PATIENTS IN WHOM A RISE OCCURRED IN THE PLATELET COUNT FROM THROMBOCYTOPENIC TO NORMAL LEVELS DURING THERAPY WITH RADIOACTIVE PHOSPHORUS

NAME	DIAGNOSIS	PLATELET COUNT AT ONSET OF THERAPY PER C.MM.	MAXIMUM PLATELET COUNT DURING THERAPY PER C.MM.
R. G.	Chronic lymphatic leukemia	77,000	347,000
J. S.	Leucosarcoma	16,000	264,000
P. S.	Leucosarcoma	62,000	498,000
D. B.	Monocytic leukemia	72,000	280,000

As shown in Table I, 41 of the 100 patients developed a leucopenia of 3,000 cells per cubic millimeter or less either during or within two months after the cessation of therapy. Here again, those patients who developed a leucopenia which was obviously due to progression of the blood dyscrasia were excluded. The figure of 41 per cent cannot be considered as absolutely accurate, as in some instances it was extremely difficult to determine with certainty whether the therapy or the disease was responsible for the depression of the leucocyte count.

A very severe neutropenia was observed in M. P., a 25-year-old female with chronic myelogenous leukemia. She was started on radioactive phosphorus therapy April 30, 1942, at which time the leucocyte count was 155,000 per cubic millimeter (Fig. 4). Within a period of thirty days she was given intravenously 9.537 millicuries of radioactive phosphorus. On the day of the last injection the white blood cell count was 23,400. Two weeks later, at a time when the leucocyte count was 14,400, she was given two additional minute doses of 0.241 and 0.160 millicuries, respectively. Thirty-six days after the last injection the white blood cell count had fallen to 950 per cubic millimeter,

of which only 9 per cent were polymorphonuclear neutrophiles. The only symptoms associated with this agranulocytosis were soreness of the left lower jaw and aching in and below the mandible. The gums appeared dirty and there was a white membrane covering the entire gingival margin. The leucocyte count gradually rose during the subsequent three weeks and the symptoms subsided.

Of the forty-one patients who developed leucopenia apparently as the result of therapy, six had definite ulceration of the mouth or nasopharynx; the other thirty-five had no specific symptoms which could be attributed directly to the leucopenia. Five of these six patients had leucosarcoma, and all had a profound neutropenia at the time therapy with radioactive phosphorus was instituted. The leucopenia which developed rapidly and dramatically in every case represented primarily a destruction of abnormal leukemic lymphocytic cells. Thus, while the rapid decrease in the leucocyte count was unquestionably due to the therapy, the ulceration of mucous membranes might well have occurred even if no treatment had been given.

The sixth patient, however, had polycythemia vera, and here both the rapidly developing leucopenia and the stomatitis were unquestionably due entirely to the therapy. This patient also developed a severe thromboctyopenia (Fig. 2). The leucocyte count prior to treatment was 43,700 cells per cubic millimeter. Twenty-four days after therapy (the equivalent of 5.67 millicuries given intravenously within a period of twenty-four hours) the white blood cell count had dropped to 1,450. In addition to the purpuric manifestations mentioned before, the patient complained of marked weakness and a severe headache. She was hospitalized and was given a blood transfusion in spite of the fact that the erythrocyte count was 6,650,000. The leucocyte count fluctuated between 1,100 and 1,900 for a period of three weeks, after which it gradually rose to normal. During this time she developed an anginal-like ulceration on the lower lip. This ulcer persisted for three weeks before finally healing.

C. *Anemia.*—In Table I it is seen that 36 of the 100 patients developed an anemia which seemed to be due to the therapy. This figure includes only those patients whose erythrocyte level dropped by one million or more cells per cubic millimeter and to a level below 3,500,000. For example, all the patients with polycythemia showed a decrease in erythrocyte count of over 1,000,000 per cubic millimeter, but only the four patients in whom the red blood cell level fell to 3,500,000 cells per cubic millimeter or less are included in the 36 per cent.

In all of the patients the fall in the erythrocyte count was gradual and usually was not manifest for several months after treatment was begun. In only a very few instances was the resultant anemia severe, but the depression of the erythrocyte count was surprisingly persistent. One patient with polycythemia vera still has an anemia eighteen months after the last injection of radioactive phosphorus.

The development of a profound anemia in a patient with polycythemia vera who was treated with moderate doses of radioactive phosphorus therapy is illustrated in Table III. It is interesting that this patient felt better at the time of her most severe anemia than she had prior to therapy, even though she also had a leucopenia and a mild thrombocytopenia. Her only symptoms at present

TABLE III

ANEMIA, LEUCOPENIA, AND MILD THROMBOCYTOPENIA IN A PATIENT WITH POLYCYTHEMIA VERA FOLLOWING THERAPY WITH RADIOACTIVE PHOSPHORUS

DATE	RADIOACTIVE PHOSPHORUS (MILLICURIES)	R.B.C. PER C.MM.	HB. (GM.)	W.B.C. PER C.MM.	PLATELETS PER C.MM.
7/14/43		8,110,000	22.3	11,000	1,233,000
7/16/43	0.979				
7/20/43	0.792	7,860,000	22.6	12,150	1,022,000
7/23/43	0.630	7,750,000	21.2	8,300	1,860,000
7/30/43	1.035	7,540,000	21.7	7,450	1,644,000
8/ 4/43	1.148	7,270,000	21.2	4,950	567,000
8/13/43	1.080	7,460,000	22.3	5,550	328,000
9/23/43		5,250,000	13.9	2,850	315,000
12/ 1/43		2,970,000	9.2	2,350	125,000
2/23/44		3,820,000	11.1	3,100	193,000
5/12/44		3,930,000	12.5	4,750	326,000

Total treatment, 5.664.

Case Summary.—R. S., a widowed female 65 years of age, first noted numbness and stiffness of her hands in the winter of 1939-1940. In January, 1941, she began to have pain in the left upper quadrant of the abdomen; she was told by a physician that this was due to an enlarged spleen. The sharp pain disappeared in a few weeks and she has had only occasional aching since. In the spring of 1942 she began to tire easily; she found it necessary to take a nap every afternoon whereas previously she had never slept during the day. She began to lose weight. In May, 1943, she noticed numbness of the feet at night. Occasionally she would wake up screaming with severe pain in her toes. She had headaches about once a week, never very severe.

Physical examination at the time treatment with radioactive phosphorus was started (July 16, 1943) revealed the following: Blood pressure was 185/115. Skin and mucous membranes were deep red in color with some cyanosis of lips, ears, and finger tips. Hair dyed (henna). The neck was stiff and there was limitation of movement in all directions as well as pain in the cervical spine. The heart was enlarged. A hard, nontender spleen was felt extending 18 cm. below the left costal margin. A sharp liver edge descended 4 cm. below the right costal margin on inspiration. There were no other abnormal findings. Weight was 115 pounds and height was 62 inches.

Laboratory Data.—For cytologic studies of peripheral blood (see Table III). Sternal aspiration of bone marrow (July 13, 1943) produced clumps of marrow which were hard and failed to spread under the weight of the cover slip in the usual manner. Microscopically, the marrow was very hyperplastic; erythroid cells as well as myeloid elements and megakaryocytes were present in increased numbers. Blood volume as determined by the method of Gibson and Evans, modified to permit use of the Evelyn photoelectric colorimeter, showed the plasma volume to be 2,380 c.c. and the total blood volume, 7,780 c.c. Blood chemistry studies were all normal. The basal metabolic rate was +15 per cent. Electrocardiogram showed right bundle branch block and occasional auricular premature contractions. The lungs were clear on fluoroscopic examination.

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are referable to severe hypertrophic osteoarthritis of the spine and arteriosclerotic heart disease (electrocardiograms show a right bundle branch block). These changes were induced by a total dose of only 5.66 millicuries of radioactive phosphorus. From the data recorded in Table IV, furthermore, it can be seen that there was a marked difference in the susceptibility of the eighteen patients with polycythemia to the toxic effects of radiophosphorus. Some subjects tolerated comparatively large doses, while others developed thrombocytopenia, leucopenia, and/or anemia when given much smaller amounts.

TABLE IV

HEMATOLOGIC DATA IN EIGHTEEN PATIENTS WITH POLYCYTHEMIA VERA TREATED WITH RADIOACTIVE PHOSPHORUS

PATIENT	RADIOACTIVE PHOSPHORUS THERAPY (MILLICURIES)	DURATION OF COURSE OF THERAPY (DAYS)	TIME FROM FIRST TREATMENT TO INITIAL DROP IN R.B.C. LEVEL (DAYS)	R.B.C. COUNT AT TIME THERAPY WAS BEGUN (M. PER C.MM.)	LOWEST R.B.C. COUNT	TIME FROM LOWEST R.B.C. COUNT UNTIL R.B.C. LEVEL WAS AGAIN TO 4,000,000 OR ABOVE (DAYS)	PLATELET COUNT AT TIME THERAPY WAS BEGUN (PER C.MM.)	LOWEST PLATELET COUNT AFTER THERAPY (PER C.MM.)	W.B.C. COUNT AT TIME THERAPY WAS BEGUN (PER C.MM.)	LOWEST W.B.C. COUNT AFTER THERAPY (PER C.MM.)
D. F.	7.03	34	40	8.59	3.29	163	2,490,000	358,000	15,450	3,400
I. McC.	5.24‡	69	40	7.33	4.77	28	264,000	230,000	18,750	4,200
	5.63	189	34	6.66	5.47	--				
C. R.	7.41	112	30	6.56*	4.59	--	3,685,000	258,000	21,600	3,950
N. W.	9.50§	2	30	7.83	3.35	31	2,036,000	29,000	43,700	1,100
O. A.	5.74	66	88	9.00	3.19	286	756,000	191,000	5,400	2,500
W. L.	8.44	70	41	6.65	4.75	†	458,000	454,000	4,600	3,200
P. R.	13.25	75	33	7.00*	3.37	138	2,080,000	287,000	13,500	1,500
R. S.	5.66	29	40	8.11	2.97	163+	1,233,000	107,000	11,100	1,200
F. V.	6.71	92	60	7.91	4.77	--	1,266,000	626,000	15,750	7,050
W. W.	10.5	49	61	7.15*	3.27	182+	2,016,000	250,000	5,000	1,650
K. H.	4.90	43	28	7.91*	4.34	--	1,818,000	164,000	11,600	4,100
M. T.	7.78	139	30	7.56	4.99	--	2,600,000	744,000	12,550	5,450
S. K.	6.92	120	47	6.47*	5.60	--	3,300,000	476,000	17,200	7,150
O. W.	5.01	87	60	8.01	5.12	--	820,000	364,000	9,900	4,300
A. B.	5.17	2	48	7.57	3.50	30+	1,582,000	153,000	9,650	950
W. M.	4.07	1	58	7.98	4.47	--	1,607,000	191,000	10,400	1,550
M. K.	4.03	1	41	6.02*	4.46	--	1,250,000	260,000	12,000	1,150
W. S.	4.14	44	83	5.78*	3.54	10+	1,543,000	724,000	13,300	5,250

*Patient treated by phlebotomies or phenylhydrazine up to the time P^{32} therapy was begun. It is probable that there was greater erythroid activity than was suggested by the red blood cell count.

†Patient not seen since July, 1943, at which time the red blood count was 4.87 million. Lowest count, 4.75 million, was in June, 1943.

‡First course of treatment (shown above) brought the red blood count down to 4.77 million. Three months after the last injection in this series of treatments, the red blood count had risen to 6.66 million and the patient was beginning to have headaches again. Second course of treatment (shown below) was therefore given.

§12.665 millicuries given orally which is equivalent to 9.50 millicuries I.V. (assuming 75 per cent absorption from the gastrointestinal tract).

Most of the patients who developed a significant anemia complained of weakness and fatigability. However, in many cases these symptoms were present prior to therapy, and it was not possible to ascertain the percentage of patients who developed symptoms as a direct result of the anemia.

D. *Aplasia of the Bone Marrow.*—At necropsy, localized areas of necrosis in the bone marrow were observed several times. In four instances, the marrow was found to be aplastic. This change occurred in the tissues of J. S., a boy 14 years of age who had leucosarcoma. When first seen, he had 4,400,000 red blood cells per cubic millimeter, 13.7 Gm. of hemoglobin, 15,600 leucocytes, and a platelet count of less than 10,000. Ninety-four per cent of the white blood cells were abnormal, immature lymphocytes. Bone marrow obtained by sternal aspiration showed an almost solid mass of leukemic cells; granulocytes and erythroid elements were rare; no megakaryocytes were seen. In the hope that therapy might lead to destruction of enough leukemia cells to permit formation of normal blood cells by the bone marrow, 6.858 millicuries of radioactive phosphorus were given over a period of twenty-eight days. Although the platelets remained low, they did rise temporarily to 44,000 per cubic millimeter. The white blood cell count, however, fell to between 100 and 500 cells, and the number of erythrocytes decreased steadily to 1,670,000 cells at the time of death, only thirty-one days after institution of therapy. The marrow at necropsy showed many areas of hemorrhage in addition to the aplasia. There were practically no morphologic evidences of leukemia in any of the tissues. Two of the other patients who developed aplasia of the marrow also had leucosarcoma, while the fourth had Hodgkin's disease. X-ray therapy had also been given in this latter instance. The four cases are similar to those studied by Kenney[7, 16] and by Diamond and Warren.[17] These observations, coupled with those which demonstrate that aplastic anemia may be produced in animals to which large doses of radioactive phosphorus are given,[15] emphasize the fact that caution must be exercised if the production of irreversible toxic effects on the marrow are to be avoided.

DISCUSSION

Description of these hematologic complications of radioactive phosphorus therapy is in no way intended to deprecate the therapeutic value of radiophosphorus. In almost all instances there was prompt recovery from the thrombocytopenias and leucopenias, with slow recovery from the anemias which had apparently been induced by the isotope. The only exceptions occurred among patients who were obviously in the terminal stages of their disease. The experience being accumulated in this clinic confirms the conclusions of other workers, that radioactive phosphorus induces excellent remissions in patients with polycythemia vera, chronic myelogenous leukemia, and chronic lymphatic leukemia. The results are at least as good as those obtained with other forms of radiation therapy; they may prove to be better. Since, however, it is important to understand the dangers as well as the limitations of any new therapeutic technique, the occurrence of these complications should receive the emphasis given to them in this report. This is particularly true because if they are recognized and further therapy is withheld, the production of irreversible damage to the hematopoietic organs may be avoided.

The higher incidence of thrombocytopenia, leucopenia, and anemia in this series of cases than in those reported from other laboratories may, in part, be explained by the fact that an attempt was made to bring the blood count of patients with leukemia to normal levels. This was done to obtain the maximum therapeutic effectiveness of the radiant energy. Most other investigators have stopped short of this goal and have preferred, rather, to treat their patients symptomatically.

As is emphasized in Table IV, however, there is a wide variation in the individual susceptibility to radioactive phosphorus therapy. The patients with polycythemia vera received doses of the material which were comparable to those used in other clinics.[10, 11] Some of the more severe complications were shown by subjects who had been given relatively small amounts of radiophosphorus, while others who were given large amounts tolerated them well. Because of the variation in susceptibility to the effects of this form of radiation therapy, dosage regulation must be individualized to a high degree. The hematologic complications may be so delayed, however, that even when great caution is exercised, they may appear weeks or months after the last dose of radioactive phosphorus has been given.

Emphasis should also be given to the fact that therapeutically induced remissions were attended by a rise in the red blood cell count of over a million cells in eleven instances and by a rise of more than 500,000 platelets per cubic millimeter in twenty-three. Two patients with subleukemic leukemia and an initial white cell count of less than 2,000 showed increases in the leucocytes to more than 6,000 (Table I). These effects are similar to those often seen after x-ray therapy.

SUMMARY AND CONCLUSIONS

1. Of 100 patients treated with radioactive phosphorus for various hematologic dyscrasias, 44 developed a thrombocytopenia of less than 100,000 per cubic millimeter, and in 33 of these it was less than 50,000; 41 showed a leucopenia of less than 3,000; and in 36 the red blood cells fell by more than 1 million cells to a level of under 3.5 million. When more than one of these changes occurred in the same individual, the white blood cells usually decreased first, the platelets second, and the red cells third. Thrombocytopenia and anemia frequently appeared as late manifestations, occurring even weeks after therapy had been discontinued.

2. Thirty-eight of the patients with thrombocytopenia appearing after therapy showed clinical manifestations of abnormal bleeding, and in one patient with polycythemia with severe neutropenia there developed an anginal-like lesion of the lower lip. Symptoms of weakness and fatigue appeared in several of the subjects whose red cell count fell to anemic levels.

3. An aplasia of the marrow was apparently induced by therapy with radioactive phosphorus in three cases of leucosarcoma and one of Hodgkin's disease.

4. As was best demonstrated in the patients with polycythemia vera, there was considerable variation in the susceptibility of different subjects to the toxic effects of radioactive phosphorus. In several instances, leucopenia, thrombocytopenia, or an anemia followed comparatively small doses of the material.

Much larger amounts were tolerated by other patients without difficulty. Dosage must be individualized to a high degree.

5. While the conclusion of other investigators, that radioactive phosphorus is a valuable therapeutic agent in the treatment of polycythemia and chronic leukemias, is confirmed, emphasis is given to the observation that severe thrombocytopenia, leucopenia, a moderate anemia, and aplasia of the bone marrow may occur as complications of the therapy. The blood of patients treated in this manner should be studied at frequent intervals so that the hematologic changes can be recognized early and further administration stopped before irreversible toxic effects on the bone marrow are produced.

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SALMONELLA FOOD INFECTION IN MILITARY PERSONNEL

An Outbreak Caused by S. oranienburg, S. typhi murium, and S. anatum

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FOOD infection is defined by Dunham[1] as an acute gastroenteritis caused by the ingestion of food contaminated with specific organisms. The paratyphoid or Salmonella group is the most common offender. Herein is described an outbreak of 115 cases of food infection in military personnel caused by three members of the Salmonella group, specifically *S. oranienburg*, *S. typhi murium*, and *S. anatum*.

CLINICAL OBSERVATIONS

On July 25, 1943, twenty-eight individuals, all males from 21 to 50 years of age, were admitted to the hospital complaining of nausea, vomiting, abdominal cramps and diarrhea of sudden onset. Bowel movements were frequent, eight to fifteen per day, loose, watery, brownish-green in color. Gross or occult blood was present in fourteen of the patients' stools. In ten specimens there was pus, three of which had considerable pus and mucus. No ova or parasites were found in any of the stools examined.

Those patients who had nausea and abdominal cramps were rid of these symptoms in 24 to 48 hours. The diarrhea lasted longer, the range in duration being 1 to 6 days, average 3 days. All patients admitted one to two days after the onset of illness had temperature elevation, 101° F. to 104° F. There was a slight leucocytosis present in the blood counts taken at this time. None of the patients was prostrated and none died.

All patients had been eating at the same mess daily. Epidemiological studies were made by investigators in an attempt to determine the cause of the outbreak. Those common foods eaten by most of the men at the evening mess were fried fish, tartar sauce, and rice pudding. At the time of the onset of symptoms, none of this food was available for laboratory examination. The fish was suspected because it had been noted that the ice refrigerating it had melted. The incubation period appeared to be 6 to 24 hours after the ingestion of the food.

In 72 hours, the first cultural reports of the stools were presumptively positive for Salmonella, and consequently eighty-seven additional men who had eaten at this mess were hospitalized. Most of this latter group had normal stools and the usual number of bowel movements. They gave a history of having had mild diarrhea prior to admission, however. (Table I.) Some who had no symptoms were later found to have positive stool cultures.

It was decided to retain these men in the hospital until 3 consecutive stool examinations were negative for Salmonella. Stools were cultured twice weekly

Received for publication, May 24, 1944.

TABLE I

SYMPTOM	NO. PATIENTS	PER CENT PATIENTS TOTAL 115
Nausea	25	21.7
Vomiting	4	3.5
Cramps	83	72.2
Diarrhea	107	93.0
Shock	0	0
Death	0	0

until the number of patients decreased sufficiently to permit examinations at more frequent intervals.

LABORATORY OBSERVATIONS

Fecal specimens were obtained in paraffin-coated sputum cups. Since the patients were in the hospital, these specimens were readily obtained and no preservative was necessary. In the laboratory they were examined as to color, character, and the presence of gross blood. Where there was no blood visible, the orthotolidine test for occult blood was performed as described by Simmons.[2] A small portion of the stool was emulsified, using physiological saline solution. This emulsion was placed on a microscope slide and examined for red blood cells, mucus, pus, ova, and parasites, especially *Endamoeba histolytica*.

A systematic method as outlined by Littman[3] was found most essential in handling the specimens involved in this outbreak. A chart was made consisting of the patients' names arranged alphabetically, dates, and bacteriological findings. After the inoculation of the culture media, all specimens were incinerated. It was deemed impractical to keep stools for restreaking as recommended by Mayfield and Gober.[4]

For bacteriological examination the following routine was employed. SS (Shigella-Salmonella) plates were heavily streaked. A portion of feces about the size of a bean was placed in selenite-F enrichment broth and both media were incubated 18 to 24 hours. At the end of this time, three to four colorless colonies or those with bull's-eye appearance on SS agar were fished and inoculated into slants of Kligler iron agar. The enrichment culture medium was streaked heavily onto SS agar. Both media were incubated 18 to 24 hours. The Kligler iron agar slants were then observed for fermentation.

Gram's stain and motility tests were made from growth on these slants. At this time, if there was a growth of a gram-negative, motile, nonsporing bacillus which produced acid and gas in the butt, hydrogen sulfide, and an alkaline slant, it was reported as follows, "Presumptive test positive for Salmonella—confirmative report to follow."

It was thought impractical to carry all cases of this outbreak to a conclusion at this point, because of the large number of specimens involved. By the selection of representative cultures from the total number of cases presumptively positive for Salmonella and employing the necessary carbohydrate fermentation and serologic tests, the etiological agents were identified.

In our bacteriological examinations, the following differential media were employed: desoxycholate-citrate agar, B.B.L.,[5] eosin-methylene blue agar, Difco[6] and SS agar, Difco.[6] The medium yielding the largest percentage of positives in our experience was SS agar. This is in accord with the findings of Mayfield

and Gober,[4] Hardy, Watt, and DeCapito,[7] Dack[8] and others. It was found that the plates of this medium could and should be heavily inoculated. They did not produce an overgrowth, however, as was noted with eosin-methylene blue agar plates. They also remained clear whereas the desoxycholate-citrate agar plates became cloudy and opaque, especially when lactose-fermenting organisms were present in any number. The good results obtained with this medium, its simplicity of preparation and economy recommend its use.

An enrichment medium such as Leifson's[9] selenite-F, B.B.L.[5] was found to be invaluable in the culturing of specimens from carriers and in those containing few enteric organisms. It is essential, however, to streak the stools initially on a plate as well as inoculating an enrichment medium because Proteus or Pseudomonas may overgrow the Salmonella when either is present. Also, when there are more than one Salmonella species present, one species may overgrow the other in this enrichment broth.

As a fermentation test medium, Kligler iron agar[6] was found to be more satisfactory than Russell double sugar agar because it contains an iron salt which is an excellent indicator of hydrogen sulfide production. The use of this medium in 3 to 4 c.c. amounts, in Wassermann or Loeffler tubes, effected a great saving of material.

Not all the carbohydrates initially employed by us were found to be essential. The use of dextrose, lactose, sucrose, maltose, mannite, inosite, tryptone water (indol), salicin, and d-sorbitol as recommended by Saphra and Silberberg[10] were found to be satisfactory for biochemical study.

Final identification was made serologically using spot group agglutinations, and then the antigenic structure of the Salmonella was determined using the Kauffmann-White schema.[11] This indentification was made in the laboratory of the New York Salmonella Center, Beth Israel Hospital, New York.

CLINICAL COURSE AND THERAPY

Patients were treated symptomatically on admission to the hospital and by the end of the first week the symptoms of gastroenteritis had subsided. When the stool cultures revealed the causative organisms to be Salmonella, treatment with sulfaguanidine (sulfanilyl guanidine) was initiated. This chemotherapeutic agent was first studied by Marshall and his colleagues,[12] and suggested for the treatment of bacterial intestinal infections. This sulfonamide is slightly water-soluble, and poorly absorbed from the intestines. A course of 16 Gm. of the drug was given to all patients between Aug. 2 and Aug. 5, 1943 (second week). That this was slightly beneficial is shown in Fig. 1, where it is noted that there was a slight decrease in the census of patients confined because of this outbreak. It is to be recalled that no patient was discharged to duty until three consecutive negative stools were obtained. A second course of sulfaguanidine, 4 Gm. per day, was given to the patients from Aug. 21 to Aug. 23 (fourth week). A few patients were found to have three negative stools following this course. It is notable that after this second administration of sulfaguanidine, *S. typhi murium* was not recovered again in any stools. There was no reaction to the drug. Blood levels of sulfaguanidine were under 1 mg. per cent.

A course of sulfasuxidine consisting of 4 Gm. daily for 3 days was given to all patients Sept. 23 to 25 (ninth week). It was noted that there was a reduction of enteric pathogens (nonlactose-fermenting organisms) on differential media. *S. anatum* was decreased markedly (see Fig. 1), while *S. oranienburg* was decreased slightly.

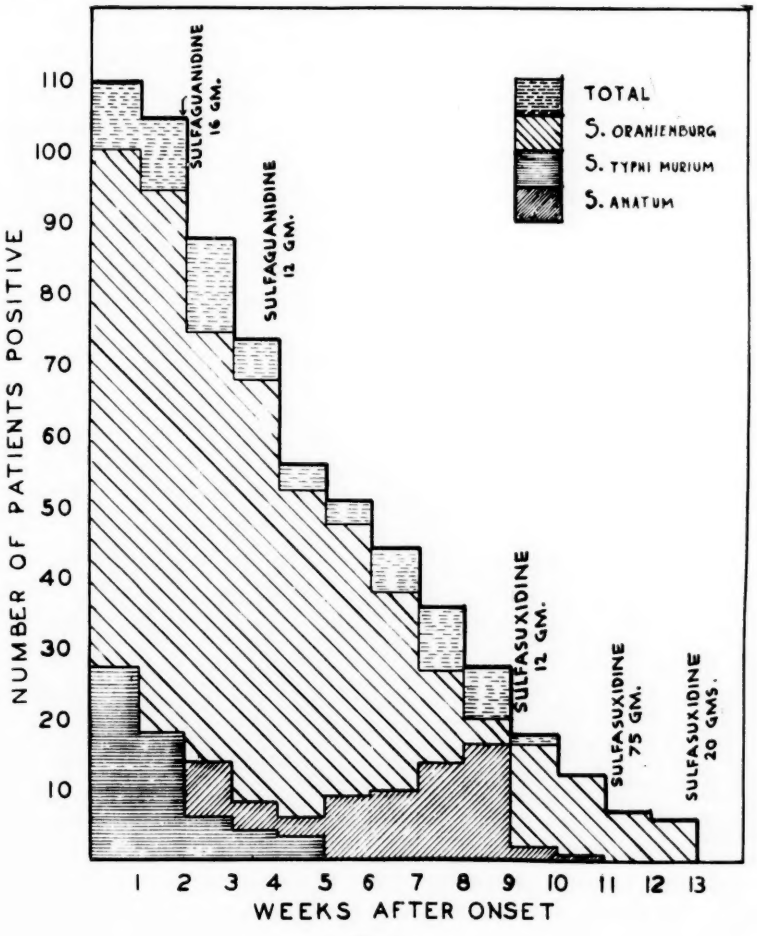

Fig. 1.

Because of the satisfactory results obtained with this dosage, the 12 remaining carriers at the eleventh week were subjected to larger quantities of this sulfonamide. Starting Oct. 13 and for 5 successive days, a total of 75 Gm. was given to 6 of these patients. All these patients' stools were free of Salmonella thereafter. The other 6 were used as controls. Two of these became negative spontaneously, while four remained persistently positive for *S. oranienburg*. These carriers were then given 20 Gm. of sulfasuxidine in 2 days and they too became Salmonella free, as determined by seven daily negative stools.

There were no toxic reactions following this sulfatherapy. Blood counts taken during this period were within the normal range. Urine analyses revealed no pathological findings. None of the patients complained of nausea, headache,

or rash. The concentration of this sulfonamide in the blood was consistently low, and usually less than 1 mg. per cent. This tends to confirm the findings of other investigators that a high concentration of this drug is maintained in the gastrointestinal tract with a low concentration of the drug in the blood.

PERSISTENCE OF SALMONELLA INFECTION

The incidence and persistence of the enteric pathogens in the 115 cases is shown in Table II. As previously stated, this outbreak was due to a multiple infection of Salmonella. The incidence was recorded and presented graphically in Fig. 1 in reference to the different species of Salmonella cultured from the patients' stools either during or subsequent to the week stated. Each patient was considered positive as long as one of the Salmonella was recovered from his stool.

During or following the first week, 109 (94.8 per cent) patients were found to have stool cultures positive for one or more of the Salmonella. *S. oranienburg* was recovered from 100 (86.9 per cent) patients, whereas *S. typhi murium* appeared in 27 (23.4 per cent). In 6 (5.2 per cent) patients the etiological agents were never recovered.

TABLE II

PERSISTENCE OF SALMONELLA INFECTION AS DETERMINED BY FECAL CULTURES IN 115 PATIENTS

WEEK FOLLOWING INGESTION OF SUSPECTED FOOD	NUMBER AND PER CENT PATIENTS POSITIVE						TOTAL NUMBER POSITIVE CASES SINGLE OR MULTIPLE	PER CENT TOTAL POSITIVE CASES SINGLE OR MULTIPLE
	S. oranienburg		*S. typhi murium*		*S. anatum*			
	NO.	%	NO.	%	NO.	%		
First	100	86.9	27	23.4	0	0	109	94.8
Second	94	81.7	18	15.6	0	0	104	90.4
Third	74	64.3	6	5.2	14	12.2	87	75.7
Fourth	68	59.1	4	3.4	8	6.9	73	63.5
Fifth	52	45.2	3	2.6	6	5.2	56	48.7
Sixth	48	41.7	0	0	9	7.8	51	44.3
Seventh	38	33.0	0	0	10	8.7	44	38.3
Eighth	27	23.5	0	0	14	12.2	36	31.3
Ninth*	20	17.4	0	0	16	13.9	28	24.3
Tenth	16	13.9	0	0	2	1.7	17	14.8
Eleventh	12	10.4	0	0	1	0.9	12	10.4
Twelfth	7	6.1	0	0	0	0	7	6.1
Thirteenth	6	5.2	0	0	0	0	6	5.2
Fourteenth	0	0	0	0	0	0	0	0

*Sulfasuxidine administered.

Table III indicates the true nature of the multiple infection. In pure culture, there were 45 (39.1 per cent) cases of *S. oranienburg*, but only 4 (3.5 per cent of *S. typhi murium* and 4 (3.5 per cent) of *S. anatum*.

There were various combinations of Salmonella in some cases. In 16 (13.9 per cent), *S. oranienburg* and *S. typhi murium* were found, in 33 (28.7 per cent), *S. oranienburg* and *S. anatum*, and 1 (0.9 per cent) had a combination of *S. typhi murium* and *S. anatum*. There were 6 cases of infection with all three species.

In summing up the incidence of each species it is noted that *S. oranienburg* was found in 100 (86.9 per cent) cases, *S. typhi murium* in 27 (23.5 per cent) and *S. anatum* in 44 (38.2 per cent) either in combination or alone.

TABLE III

INCIDENCE OF SALMONELLA INFECTION SINGLE AND MULTIPLE

ENTERIC PATHOGEN	NUMBER PATIENTS POSITIVE	PER CENT PATIENTS POSITIVE
Single:		
S. oranienburg	45	39.1
S. typhi murium	4	3.5
S. anatum	4	3.5
Multiple:		
S. oranienburg + *S. typhi murium*	16	13.9
S. oranienburg + *S. anatum*	33	28.7
S. typhi murium + *S. anatum*	1	0.9
S. oranienburg + *S. typhi murium* + *S. anatum*	6	5.2
Negative	6	5.2
Total	115	100.0

SEROLOGIC OBSERVATIONS

It is noteworthy that all these patients had received a course of triple typhoid vaccine (TAB) containing *E. typhosa*, *S. paratyphi* (para A) and *S. schottmuelleri* (para B).

Initial bloods were collected on Aug. 6, 2 weeks after onset (see Table IV), and tube agglutination tests were performed at 56° C. for 2 hours, using homologous cultures of *S. typhi murium*, *S. oranienburg* or both.* *S. anatum* was not included at this time, because this organism had not yet been recovered from the stools (see Table II). All of the 23 patients from whom blood was drawn had symptoms, and their stool cultures were positive for one or both of the Salmonella. Four of the five sera tested with *S. typhi murium* showed no agglutination titer, although this organism was recovered from the stools (Table IV, Nos. 7, 9, 12, 14). The remaining one showed a 2+ titer at 1:200 dilution (No. 23). When the bloods were tested with the homologous antigen *S. oranienburg*, however, 18 showed a definite antibody titer, 1:3200 in some instances (Nos. 11 and 12), and the majority agglutinated at 1:200. A few specimens had lower titers, 1:50 and 1:100. In all instances where *S. oranienburg* was recovered from the stool, the homologous antibody was present in the patient's blood at the end of this 2-week period. In the two cases where *S. oranienburg* and *S. typhi murium* were recovered from the stools (Nos. 12 and 14), the bloods titrated with the homologous antigens showed strong titer for *S. oranienburg*, 1:3200 and 1:800, while they were negative for *S. typhi murium* in all dilutions.

Thus, at the end of the second week, it was possible to confirm the diagnosis of Salmonella infection by means of blood agglutination tests.

The second blood specimens were collected from 28 patients on Aug. 14 and titrated with the apparently predominant organism, *S. oranienburg*. Nine

*These tests were performed by M/Sgt. Robert J. Helmold of the Bacteriology Department of the Second Service Command Laboratory.

TABLE IV

SEROLOGIC DATA

(Aug. 6, 1943)

PATIENT	SALMONELLA RECOVERED IN STOOL AND ANTIGEN USED	BLOOD TITER (2 HR. @ 56° C.) SERUM DILUTIONS						
		1/50	1/100	1/200	1/400	1/800	1/1600	1/3200
1	*S. oranienburg*	2+	2+	0	0	0	0	0
2	*S. oranienburg*	3+	3+	0	0	0	0	0
3	*S. oranienburg*	4+	4+	4+	4+	0	0	0
4	*S. oranienburg*	4+	4+	2+	0	0	0	0
5	*S. oranienburg*	4+	4+	4+	4+	4+	0	0
6	*S. oranienburg*	4+	4+	4+	0	0	0	0
7	*S. typhi murium*	0	0	0	0	0	0	0
8	*S. oranienburg*	2+	2+	0	0	0	0	0
9	*S. typhi murium*	0	0	0	0	0	0	0
10	*S. oranienburg*	4+	4+	4+	2+	0	0	0
11	*S. oranienburg*	4+	4+	4+	4+	4+	4+	3+
12	*S. oranienburg*	4+	4+	4+	4+	4+	4+	2+
	S. typhi murium	0	0	0	0	0	0	0
13	*S. oranienburg*	4+	4+	4+	4+	3+	0	0
14	*S. oranienburg*	4+	4+	4+	4+	2+	0	0
	S. typhi murium	0	0	0	0	0	0	0
15	*S. oranienburg*	4+	4+	2+	0	0	0	0
16	*S. oranienburg*	2+	1+	0	0	0	0	0
17	*S. oranienburg*	4+	4+	4+	4+	4+	2+	0
18	*S. oranienburg*	2+	0	0	0	0	0	0
19	*S. oranienburg*	2+	2+	0	0	0	0	0
20	*S. oranienburg*	4+	4+	4+	4+	4+	2+	0
21	*S. oranienburg*	4+	4+	4+	4+	2+	0	0
22	*S. oranienburg*	4+	4+	4+	4+	2+	0	0
23	*S. typhi murium*	3+	3+	2+	0	0	0	0

4+—coarse flocculation—clear supernatant fluid
3+—coarse flocculation—slightly turbid fluid
2+—fine flocculation—moderately turbid fluid
1+—very fine flocculation—moderately turbid fluid
0—no flocculation—opalescent fluid

of these specimens were from patients whose sera had been tested previously. It was found that there was a persistence and in some instances an increase in antibody titer. In the remaining nineteen new specimens drawn from patients whose stools were known to be positive for this bacillus, definite titers were obtained, varying from 1:100 to 1:3200, the majority being 1:200.

Number of bloods tested previously and now repeated	9
Persistence of titer	4
Increase	3
Slight decrease	2

The third blood specimens were drawn from 69 patients on Aug. 24 (fourth week). They were now tested with *S. oranienburg*, *S. typhi murium* and *S. anatum*, since one or more of these pathogens had been recovered from the patients' stools. Thirty-two of these patients had had at least one previous agglutination test for *S. oranienburg*, with the following results:

Number of bloods tested previously and now repeated	32
Persistence of titer	14
Increase	3
Decrease	15

Sera from the other 37 patients gave a definite agglutination titer for *S. oranienburg* ranging from 1:50 to 1:1600. Patients from whom *S. anatum* and *S. typhi murium* had been recovered showed little or no titer for these organisms. Two cases in which only *S. typhi murium* was found in the stools showed a positive titer for *S. oranienburg* in the patients' blood. In a few instances *S. typhi murium* antibody was 1:50 or 1:100, which may be attributed in part to the TAB vaccine, since *S. schottmuelleri* has a similar somatic antigenic structure.

On Sept. 25 (two months), a group of 31 blood sera was tested. All were titrated against *S. oranienburg,* because, as previously noted, this was the one organism which produced definite antibody reaction.

Number of bloods previously tested and now repeated	26
Persistence in titer	8
Increase	3
Decrease	15

Eighteen of these sera still had a relatively high titer for this Salmonella, about 1:400 and as high as 1:3200 in some instances. There were 2 cases in which *S. anatum* was the only organism recovered in the stool. Their blood sera, however, showed a high titer, 1:1600 for *S. oranienburg,* again proving the multiplicity of the infection. Sera from the 5 patients tested for the first time gave a definite agglutination titer for *S. oranienburg.*

On Oct. 22, 9 blood sera were examined for antibody titer with *S. oranienburg.* All showed a decrease in titer, although in some it persisted, 1:400.

Number of bloods previously tested and now repeated	9
Persistence in titer	0
Increase	0
Decrease	7
Decrease to negative	2

In conclusion, a rapid titer was produced at the end of the second week for *S. oranienburg,* slight or no titer for *S. typhi murium* and *S. anatum.* There was a gradual decrease and at the end of three months there was but weak antibody present, in some instances none.

DISCUSSION

There have been several reports of multiple enteric infections described in the literature, especially in reference to epidemics. Edwards and Bruner[13] noted multiple infections caused by various paratyphoid bacilli in fowl. Bornstein,[14] Hormaeche, et al.[15] and Schiff and Saphra[16] described human cases of food infection in which more than one species of Salmonella were recovered in the stools. That this is also the case in outbreaks of dysentery has been stated by Topley and Wilson[17] and by Hardy and Watt.[18] In this outbreak three distinct species were involved, *S. oranienburg, S. typhi murium,* and *S. anatum.*

S. oranienburg was found in 100 of the cases, by far the greatest incidence of specific Salmonella. Because of this and the positive blood serum agglutinations obtained with this organism, it may be considered the most important etiological agent. It is evident, however, in this outbreak, that all three Salmonella were originally ingested with the contaminated food.

S. oranienburg was first isolated from the stool of a healthy boy and described by Kauffmann[19] in 1930. The Kauffmann-White schema[11] lists this organism in group C as having the following antigenic structure: $VI_1.VI_2.VII$: m,t, :—.

In 1936, Edwards[20] described a case of *S. oranienburg* in quail. Schiff and Strauss,[21] in 1939, identified this organism in the feces of a three-year-old child with gastroenteritis, the first such case to be recorded in the United States.

S. anatum was first isolated from ducklings by Rettger and Scoville[22] in 1919. It is placed in group E and its antigenic formula is III.X.XXVI: e,h : 1,6. Bergey, et al.[23] state that *S. anatum* frequently occurs in association with *S. typhi murium*.

S. typhi murium, a natural pathogen of rodents, was first isolated from a mouse in 1890 by Loeffler and given the name of *B. typhi murium*. In 1898, DeNobele[24] recorded a case of gastroenteritis caused by this organism under the name of *S. aertrycke*. The Kauffmann-White schema places this organism in group B with the antigenic structure [I].IV.[V].XII : i : 1,2,3. With the exception of *S. schottmuelleri*, it is the most widely distributed of all the Salmonella.[25]

Edwards and Bruner[26] have demonstrated that each one of the Salmonella found in this outbreak can produce symptoms in most warm-blooded animals.

Clinically, the course of the disease in all cases was of short duration. The symptoms of gastroenteritis disappeared, but the stool cultures were positive for one or more Salmonella at repeated intervals. It is notable that in the 6 cases in which all three species were found none was more acutely ill than any other case. When Salmonella invade the blood stream, however, the symptomatology becomes much more acute. Jager and Lamb[27] reported 2 cases of *S. oranienburg* bacteremia. Since the infection involved in this outbreak was apparently confined to the gastrointestinal tract, blood cultures were not taken.

The importance of serologic tests using the patients' homologous antigen proved of great value in our study. The titer for *S. oranienburg* was high and persistent. At no time was there a significant titer for *S. typhi murium* or *S. anatum*. Thus, *S. oranienburg* was the one organism which caused an antibody reaction. Mosher, et al.[28] observed no significant antibody titer in patients involved in an outbreak of enteritis due to *S. typhi murium*. Kross and Schiff,[29] however, found an agglutination titer of 1:1,600 in a case of *S. typhi murium* enteritis which simulated appendicitis. Other authors have found blood titers for members of the Salmonella group, although it appears that a titer for specific agglutinin is not always produced following an enteric Salmonella infection. Jager and Lamb[27] reported a titer for *S. oranienburg*.

The occurrence of Salmonella carriers has been described by Rubenstein, Feemster and Smith.[30] Mosher, et al.[28] demonstrated *S. typhi murium* in stools of convalescent carriers for eighteen weeks. In our cases *S. typhi murium* was not recovered after the fifth week. This may be due to the influence of sulfa-

guanidine or to the mechanical removal of the organisms in the course of time. *S. anatum* was first identified in the stools during the third week. In all, forty-four patients were found to harbor this organism. Since the patients were isolated and the organism was obtained from patients on different wards, secondary contamination was unlikely. The preponderance of *S. oranienburg* and *S. typhi* murium colonies, however, increased the likelihood of these organisms being fished and studied for identification. The possibility of fewer *S. anatum* organisms originally ingested should not be overlooked. Dack[8] states that the smaller the number of organisms swallowed, the longer the incubation period, and that during the acute symptoms one may obtain practically pure cultures. *S. anatum* persisted for eleven weeks, and *S. oranienburg* for thirteen weeks.

The carrier state is a source of danger to the individual harboring the organism and as the origin of outbreaks. It was with this in mind and in reference to the increased hazard where persons have been congregated that these patients were kept in the hospital until three consecutive negative stools were obtained. The actual hazard has been demonstrated by Hardy and Watt[31] in the case of fingernail studies in which Shigella organisms were recovered. Fingernail studies performed on twelve carriers positive for *S. oranienburg* were negative.

Two sulfonamides were employed in an attempt to eliminate these pathogens. Sulfaguanidine was administered early in the course of the infection and may have been effective in the removal of *S. typhi murium*. There was little or no effect noted with regard to the number of patients whose stools were persistently positive for *S. oranienburg* and *S. anatum*.

When succinyl sulfathiazole was given these patients, the convalescent carrier state was markedly curtailed. Kirby and Rantz[32] used this sulfonamide with good results in the treatment of dysentery carriers, but found it to be of no value in the treatment of typhoid carriers. Poth, et al.[33] reported prompt recovery of bacillary dysentery carriers following the administration of sulfasuxidine in small dosages. At the end of the first course of this drug, consisting of 12 Gm. over a period of three days, nearly all of the patients' stools were cleared of *S. anatum*. This small dose was insufficient to produce a marked effect on *S. oranienburg*. Barker[34] recommends a dosage of 0.25 Gm. per kilogram for 5 to 7 days to eliminate dysentery bacilli in the stools of carriers. Following the second course of succinyl sulfathiazole, consisting of 75 Gm. over a 5-day period, there was a notable decrease of *S. oranienburg* carriers. The bacteriostatic effect of this sulfonamide was noted culturally and is presented graphically in Fig. 1.

SUMMARY

1. An outbreak is reported involving 115 cases of acute gastroenteritis in military personnel eating at the same mess.

2. The causative organisms identified on stool culture were *S. oranienburg* in 100 (86.9 per cent) cases, *S. typhi murium* in 27 (23.5 per cent) and *S. anatum* in 44 (38.2 per cent).

3. The convalescent carrier nature of the organisms was determined by frequent stool examinations. *S. oranienburg* persisted for 13 weeks, *S. anatum* for 11 weeks and *S. typhi murium* for 5 weeks.

4. Etiological agents were identified culturally, biochemically, and serologically.

5. A high antibody titer for *S. oranienburg* was demonstrated by blood serum agglutinations.

6. Succinyl sulfathiazole in adequate dosage had a bacteriostatic effect on *S. oranienburg* and *S. anatum*, aiding in the termination of the carrier state.

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A STUDY OF THE CHEMICAL COMPOSITION AND ANTIGENIC PROPERTIES OF A POLYSACCHARIDE FRACTION OF THREE-HOUR CULTURES OF STAPHYLOCOCCUS AUREUS

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SEVERAL studies have been made of the chemical composition and antigenic properties of fractions derived from the staphylococcus. Goadby[1, 2] separated several different protein and carbohydrate fractions from this organism and studied them superficially without particular attention to chemical structure. Julianelle and Wieghard[3] made a more intensive study of the carbohydrate fraction with respect to its nature and antigenic ability. In this study on both smooth and rough strains, these authors were unable to define the sugar resulting from hydrolysis of the isolated carbohydrate fraction but found it to be a hapten rather than a complete antigen and indicated it as the determinant of type specificity. Hoffstadt and Clark[4] have made an extensive study of the chemical composition of the *Staphylococcus aureus* and tested the antigenic capacity of the various fractions. Those fractions giving the Molisch test induced complement-fixing antibodies, and two of these carbohydrate-containing substances produced precipitins. No attempt was made to define any of these fractions chemically or to determine their place in the antigenic structure of the organism. Verwey[5] by a chemical extraction of Type A staphylococci found a type-specific antigenic fraction which was a protein. This fraction was independent of the type-specific carbohydrate described by Julianelle and Wieghard.[3]

In attempting to repeat some of the experiments published by Champ Lyons,[6] we noted, as he had, that at three hours, cultures of *Staphylococcus aureus* have a larger halo about them than at any other period of their growth. Antisera prepared from these cultures gave good protection in mice (Fellowes[7]). Swiss mice in groups of 20 were given fatal doses of the staphylococci and two hours after infection as little as 0.3 c.c. of antiserum. All of the control group died and 14 (average) of the experimental group survived. This led to the belief that the halo about the organism might have something to do with the antibodies produced by such cultures and might be chemically defined.

PREPARATION OF CULTURES

Each of two strains of a pathogenic *Staphylococcus aureus*, IC and CB,* was grown in 500 c.c. lots of dextrose meat infusion broth for three hours. These cultures were centrifuged and washed repeatedly with physiological saline to remove all traces of medium and were then dried in acetone to a white powder and preserved in the cold room until used. This process was

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Received for publication, May 12, 1944.

*Iowa City and Council Bluffs.

slow and several years were required to accumulate enough dried organisms to proceed with chemical extraction and analysis. For information on the nitrogenous constituents of the organism, the nitrogen distribution in the dried bacteria was determined by the Cavett[8] modification of the Van Slyke[9] procedure. The ash content was determined by heating to redness in a muffle furnace for six hours. Phosphates were estimated by the Fiske and Subbarow[10] method on acid solutions of the ash. The colorimetric method of Shinohara[11] was used to determine cystine, and the Benedict-Denis method was used for total sulfur. The average values obtained are shown in Table I.

TABLE I

BACTERIAL ANALYSES

	IC STRAIN* PER CENT OF TOTAL N	CB STRAIN† PER CENT OF TOTAL N	IC STRAIN* PER CENT	CB STRAIN† PER CENT
Amide N	13.16	13.98		
Humin N	4.17	4.90		
Dibasic N	29.52	29.97		
Arginine	8.55	8.26		
Histidine	9.94	11.25		
Lysine	10.45	9.87		
Cystine	0.58	0.59		
Mono- and non-amino N	53.61	51.78		
Amino N	48.10	45.10		
Total N accounted for	100.46	100.63		
Ash			13.91	13.60
Phosphorus			1.85	1.80
Sulfur			0.45	0.47
Nitrogen			13.18	12.79

*Iowa City strain.
†Council Bluffs strain.

EXTRACTION PROCESS

In preliminary experiments phenol and trichloracetic acid were found unsatisfactory as solvents for isolating the carbohydrate from the dried ground organisms. Diethylene glycol, which had been tried by Morgan,[12] gave good results until dialysis with cellophane was attempted; the carbohydrate passed through the cellophane casing and the yield was lost in the dialysate. Fuller's method[13] for obtaining polysaccharides from streptococci, employing formamide as the solvent, was found satisfactory.

The dried ground bacteria, usually in 5 or 10 Gm. portions, were heated with 15 volumes of formamide at 150° C. for 20 minutes. After cooling, 2½ volumes of acidified alcohol were added, the precipitate was filtered off and re-extracted twice with 70 per cent alcohol. The combined alcoholic extracts were mixed with an equal volume of acetone and the precipitate which contained the extracted carbohydrate was separated by centrifugation and dissolved in a small amount of water. A portion remained undissolved even after small amounts of dilute acid were added. This insoluble fraction was largely nucleoprotein.

Further separation of the water-soluble material was effected by fractional precipitation with acidified acetone. Two to 5 volumes of acetone precipitated most of the carbohydrate. Addition of 15 volumes of acetone to the supernatant liquid produced a surprisingly large precipitate. Thus three fractions were obtained: nucleoprotein, carbohydrate, and the 15-volume acetone precipitate.

CHEMICAL STUDIES

Chemical studies on the above fractions were confined to the carbohydrate and the 15-volume acetone precipitate, since the amount of nucleoprotein was insufficient for analysis.

Aqueous solutions of the carbohydrate did not reduce Benedict's solution until after hydrolysis. The biuret test was weakly positive. The iodine test for glycogen was negative; the Molisch test was positive in every case. Positive tests for pentose were given by Bial's and the naphthoresorcinol test. According to Tollens' naphthoresorcinol test glycuronic acid was not present. Methyl pentoses were not present as shown by a negative response to the ammonium molybdate test, the Tollens and Rorive modification of the naphthoresorcinol test and spectral reactions. Characteristic osazone crystals were formed as irregular rosettes with smooth broad petals; they were soluble in hot water. These osazones could not be identified with those prepared from pure specimens of several pentoses and hexoses.

Sugar analyses were made by the Somogyi method[14] with the microsugar reagent. Hydrolysis of 5 to 10 mg. samples was carried out by gentle boiling of the mixture on a sand bath under a reflux with 5 c.c. of 0.5N HCl for five to eight hours. The method of Somogyi[15] was used for the determination of fermentable and nonfermentable sugar. Larger samples of the polysaccharide (15 to 30 mg.) were hydrolyzed for this procedure.

Nitrogen was estimated by a semimacro-Kjeldahl method on 5 to 10 mg. of material. Amino nitrogen was determined by the Kendrick and Hanke modifications[16, 17] of the Van Slyke method on aliquots of acid hydrolysates (0.5N HCl) containing 5 mg. of the polysaccharides.

Ash was determined by igniting 20 mg. samples at bright red heat for six hours in a muffle furnace. Phosphates were estimated by the Fiske and Subbarow[10] method on acid solutions of the ash.

The specific rotation of the preparations was measured on aqueous solutions which were then made 0.5N by the addition of hydrochloric acid and hydrolyzed for six hours, as described previously.

Equivalent weight was obtained by titration of 20 mg. samples with 0.01N NaOH and phenolphthalein as the indicator. The samples were dissolved in approximately 1 c.c. of water.

Since the qualitative tests for pentose and uronic acid are so closely related, a modification of the method of Bowman and McKinnis[18] for the simultaneous determination of pentose and uronic acid was used in the quantitative estimation. The furfural that was formed was measured by Heidelberger's modification[19] of the Pervier and Gortner method.[20] Uronic acid was not present in the polysaccharides since the formation of barium carbonate never exceeded that of a blank determination. Samples of arabinose (10 mg.) were analyzed for their pentose content by this method. Values of from 9.77 to 10.26 mg. were obtained in six determinations.

The glucosamine content of the polysaccharides was estimated by a combination of the methods of Palmer, Smyth, and Meyer,[21] and Elson and Morgan.[22]

The average values for some of the preparations are shown in Table II.

TABLE II

POLYSACCHARIDE ANALYSES

	IC STRAIN		CB STRAIN		15-VOLUME ACETONE PRECIPITATE
	FROM 10 GM.	FROM 5 GM.	FROM 10 GM.	FROM 5 GM.	
	%	%	%	%	%
Reducing sugar after hydrolysis	18.27	21.50	19.20	19.84	1.50*
Nonfermentable sugar	15.82	18.60	17.48	17.60	1.15
Fermentable sugar	2.45	2.90	1.72	2.24	0.35
Pentose	17.13	18.97	19.61	18.68	6.16
Total nitrogen	8.18	7.08	6.50	6.92	21.40
Amino nitrogen	3.07	2.41	2.99	2.47	9.55
Per cent of total nitrogen	37.50	34.10	46.00	35.70	44.60
Glucosamine	8.97	7.20	6.70	9.87	4.63
Ash	2.44	2.84	4.63	3.17	3.05
Phosphorus	1.06	1.22	1.09	1.00	0.83
Acid equivalent N/100 NaOH	463.00	370.00	467.00	379.00	657.00
$[\alpha]_D^{27}$ before hydrolysis	+8.02	−8.60	−2.81	+7.63	−29.30†
$[\alpha]_D^{27}$ after hydrolysis	+15.27	+12.93	+11.70	+14.11	+33.70†

*Hydrolyzed with 4N HCl.

†$[\alpha]_D^{35}$

TABLE III

FLOCCULATION TESTS OF CARBOHYDRATE FRACTIONS WITH THEIR ANTISERA

CARBOHYDRATE ANTISERA	DILUTION OF ANTIGEN				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
			IC antigen		
IC-1	+	+	+	−	−
IC-2	+	+	+	−	−
Normal serum	−	−	−	−	−
			CB antigen		
CB-1	+	+	+	−	−
CB-2	+	+	+	−	−
Normal serum	−	−	−	−	−

ANTIGENIC REACTIONS

So little of the carbohydrate was left after the chemical investigation was finished that amounts used and numbers of animals employed were limited. Two rabbits were inoculated with each of the polysaccharides once a week. The initial dose was 1 mg.; in each of the next four weeks they received 2 mg., and after a rest of one week each received 1 mg. This made 10 mg. of carbohydrate that each animal received. The rabbits were bled the seventh week.

Flocculation tests were again used to determine the results of the immunization. Tenfold dilutions of each of the polysaccharide preparations were made and the antiserum held constant at a 1:5 dilution. The two components were used in 0.5 c.c. amounts. The tests were incubated in the cold room overnight and at room temperature for a few hours the next day. The results appear in Table III.

One month after immunization the animals were bled and tested again. These results were more promising and appear in Table IV.

There were available some antisera prepared by injection of 3-hour cultures of whole organisms of other strains of pathogenic staphylococci including the two strains investigated here. These strains were designated IC, CB, X, and Y. Flocculation tests were set up with the diluted polysaccharide antigens from IC and CB strains and antiserum to all four strains of whole organism.

TABLE IV

FLOCCULATION TESTS OF CARBOHYDRATE FRACTIONS WITH THEIR ANTISERA*

CARBOHYDRATE ANTISERA	DILUTION OF ANTIGEN 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
			IC antigen		
IC-1	+	+	+	+	–
IC-2	+	+	+	+	–
Normal serum	–	–	–	–	–
			CB antigen		
CB-1	+	+	+	+	–
CB-2	+	+	+	–	–
Normal serum	–	–	–	–	–

*These antisera were taken 30 days after last immunization of animals.

TABLE V

FLOCCULATION TESTS OF CARBOHYDRATE FRACTIONS WITH VARIOUS ANTISERA PREPARED BY USE OF WHOLE ORGANISMS AS ANTIGEN

CARBOHYDRATE ANTISERA	DILUTION OF ANTIGEN 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
				IC antigen			
IC	+	+	+	+	+	+	–
CB	+	+	+	+	+	+	–
X	+	+	+	+	+	+	–
Y	+	+	+	+	+	+	–
Normal serum	–	–	–	–	–	–	–
				CB antigen			
IC	+	+	+	+	+	+	–
CB	+	+	+	+	+	+	–
X	+	+	+	+	+	+	–
Y	+	+	+	+	+	+	–
Normal serum	–	–	–	–	–	–	–

TABLE VI

FLOCCULATION TESTS OF SUPERNATANT OF CULTURES WITH ANTISERUM TO CARBOHYDRATE FRACTIONS

CARBOHYDRATE ANTISERA	DILUTION OF SUPERNATANT 10^{-1}	10^{-2}	10^{-3}	10^{-4}
		IC supernatant		
IC-1	+	+	+	–
IC-2	+	+	+	–
Normal serum	–	–	–	–
		CB supernatant		
CB-1	+	+	–	–
CB-2	+	+	–	–
Normal serum	–	–	–	–

These antisera had been preserved in sealed ampoules for more than six months at time of use. The outcome of these tests is found in Table V.

Next the supernatant of the cultures used for producing the polysaccharide was tested against the antiserum to the polysaccharide. Dilutions of the supernatant of 24-hour cultures of IC and CB in dextrose meat infusion broth were diluted from 10^{-1} to 10^{-5} and the antisera were used without dilution. Equal volumes of the components were employed and the same incubation procedures utilized previously were followed. These findings are recorded in Table VI.

DISCUSSION

Based on the analysis of whole bacteria, there appeared to be no demonstrable difference between the two strains of staphylococci.

The method of preparation of the polysaccharide does not appear to produce a protein-free product. A slight biuret test, however, does not indicate a large amount of protein. The rather high nitrogen content may indicate contamination of the product by protein. On the other hand, the amino nitrogen accounts for approximately 40 per cent of the total nitrogen which would suggest nitrogen-containing material other than protein. The amino sugar present could account for only a small proportion of the total amino nitrogen unless it was altered in the process of preparation or hydrolysis.

The values obtained for reducing sugar after hydrolysis and for fermentable and nonfermentable sugars are in close agreement with those reported by Julianelle and Wieghard[3] for a pathogenic strain of staphylococcus. The presence of a large proportion of nonfermentable reducing sugar suggested the possibility of a pentose, but the above authors reported a negative qualitative test for this substance. Every preparation of the carbohydrate in this investigation gave strongly positive pentose tests. As little as 0.03 mg. gave a positive Bial's test. The values obtained in the quantitative estimation of pentoses accounted for slightly more than the nonfermentable sugar of the polysaccharide. It is not improbable that the amino sugar may be present as an amino pentose.

The polysaccharides were characterized by a low dextrorotation after hydrolysis. The material that was precipitated by 15 volumes of acetone had a higher rotation with little change on hydrolysis. This material gave abnormally high total and amino nitrogen values and in every case gave a value for pentose although it exhibited no reducing sugar after hydrolysis with 0.5N HCl. When hydrolyzed with 4N HCl this preparation yielded a small quantity of reducing sugar which was mainly nonfermentable.

Experiments on the antigenic capacity of the carbohydrate preparations were of necessity limited because of the small amount of the polysaccharide available. It was possible to show that a relationship existed between the polysaccharide antigen and antisera developed against the whole organism. Four different antisera to the whole organism of the pathogenic group reacted with each of the carbohydrate preparations to at least a dilution of 1:1,000,000 of the antigen. The reaction might have been even more delicate if the antisera had been fresh.

The reaction between the polysaccharide antigen and its antiserum produced in rabbits indicated that the carbohydrate was antigenic to some degree. A flocculation test set up immediately after the completion of immunization showed that the antigen could be diluted at least to 1:1,000 and give a visible reaction with the antiserum. The antigen has nitrogen in its complex but, as discussed previously, it is not determined in what form it occurs, whether as a contaminating protein or as amino groups in the sugar molecule. When the sera from the immunized animals were again tested after thirty days, they demonstrated an increased antibody content; dilutions of 1:10,000 gave flocculation.

It was interesting to find that cultures of these pathogenic staphylococci gave off to the medium sufficient of the antigenic substance to react with the carbohydrate antiserum. This reaction could possibly be used in diagnostic

procedures to distinguish between pathogenic and nonpathogenic strains. Julianelle and Wieghard[3] have already shown that there is an antigenic difference between the two divisions.

SUMMARY

1. A polysaccharide, containing some nitrogen, has been obtained from pathogenic strains of staphylococci by use of formamide as an extractive.

2. This polysaccharide yields a pentose on hydrolysis. The osazone crystals of this pentose have not been identified.

3. The polysaccharide is antigenic and reacts in flocculation tests with its own antiserum, and with antisera against whole organisms.

4. The culture medium in which these strains of pathogenic staphylococci were grown contained enough polysaccharide to give a flocculation test with the antisera produced with the pure polysaccharide.

5. There were no demonstrable differences between the two strains of staphylococci used.

The authors wish to thank Dr. W. M. Hale, chairman of the Department of Bacteriology, University of Iowa, for facilities, media, and counsel in the accomplishment of a part of this study in that department.

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SERIAL BIOPSY STUDIES OF THE EFFECTS OF ESTROGENS ON THE LIVER

Chemical and Morphologic Responses to Diethylstilbestrol and Estradiol

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SINCE the introduction of the synthetic estrogen, diethylstilbestrol, a number of reports on the estrogenic potency and the toxicology of this substance have appeared. In some of the investigations the properties of the synthetic estrogen were compared with the properties of the estrogens derived from natural sources. It is apparent from these studies that the quality of estrogenic activity of diethylstilbestrol is comparable to that of the natural estrogens. Furthermore, it has been shown in animals that these two different types of estrogenic substances are capable of producing similar possibly toxic changes in various organs and tissues.[1] Because an appreciable number of women receiving the synthetic estrogen diethylsilbestrol have experienced nausea and vomiting, and since in a few cases it has been suspected of causing jaundice, attention has been focused on the liver. No definite evidence has been produced by studies of hepatic function in man to show that diethylstilbestrol damages the liver. The results obtained following the administration of diethylstilbestrol to animals permit divergent conclusions in regard to the effect on the liver.[2-6] The hepatic changes have been variously interpreted as fatty degeneration and hydropic degeneration, fatty degeneration and toxic necrosis, hepatitis, and glycogen deposition. Some of the papers include reports of parallel studies with estrogens derived from natural sources, with similar results.

In considering the toxicity both of diethylstilbestrol and of the natural estrogens, it must be recognized that the dosage per unit of body weight employed in many of the experiments on animals exceeded the therapeutic range used in man. Furthermore, there are apparent differences in species susceptibility to these substances. An alteration of the bone marrow and the formed elements of the peripheral blood is easily produced by the administration to dogs of large doses either of diethylstilbestrol or of the natural estrogens.[1, 7] Similar changes are much more difficult to produce in the rat[8] and have not been observed in the monkey.[9, 10] Therefore it seems possible that estrogenic substances might influence the histologic picture and the metabolism of the liver of different species of animals in different manners. In a previous study[5] two of us gave large doses of natural and synthetic estrogens to dogs. Hepatic changes were caused by both estrogens, consisting of mild fatty degeneration and hydropic degeneration. There was considerable variation in the amount of glycogen

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Received for publication July 29, 1944.

present in different livers as determined by Best's carmine stain. No correlation could be established between the degree of fatty degeneration and hydropic degeneration and the amount of glycogen present. Teague,[6] however, has shown that the amount of glycogen in the livers of rats increases following the administration of estrogenic substances. To determine more accurately the nature of the changes observed in the livers of dogs following the administration of estrogens, we have done chemical and histological studies on samples of liver obtained both before and after the administration of diethylstilbestrol and of estradiol.*

MATERIAL AND METHODS

The studies reported were performed on eight healthy, mature dogs, 5 females and 3 males, weighing from 6 to 19 kilograms. Four of the animals, 3 females and 1 male, were used as controls. The dogs were given a diet of Purina Dog Chow. Complete counts of the formed elements of the peripheral blood were made two or three times a week.

At the termination of the control period each animal was weighed (fasting), and blood was drawn from the external jugular vein for determinations of the hematocrit, total protein, albumin, and globulin. Each animal was then anesthetized by intravenous sodium pentobarbital (0.5 mg. per kilogram of body weight). After ligation of the end of an accessible lobe of the liver a small specimen was quickly excised. One part of the specimen was used for quantitative determinations of glycogen, fat and total nitrogen and another part was used for histologic studies. Small samples of tissue were fixed in absolute alcohol and examined for glycogen by Best's carmine stain, and in solution of formaldehyde U.S.P. diluted 1:10 and examined for fat by the scarlet red stain. In addition comparable samples of tissue were fixed in a solution of Zenker's acetic acid and stained with eosin and methylene blue (methylthionine chloride U.S.P.).

The animals were divided into four groups of two each according to the type of intramuscular injections given. A dose of 1.66 mg. of estradiol dipropionate was considered to be approximately equivalent estrogenically to 5 milligrams of diethylstilbestrol dipropionate.

Group I—One male and one female dog, numbers 1 and 2, were given daily injections of 5 mg. of diethylstilbestrol dipropionate in 1 c.c. of olive oil. On the seventeenth day of the injections, a count of the blood disclosed a moderate thrombocytopenia. On the following morning biopsy specimens of the liver were obtained, and immediately thereafter complete autopsies were performed.

Group II—Dogs 3 and 4, male and female respectively, served as the control animals for the Group I dogs. Each dog received 1 c.c. of sterile olive oil for 17 days, then biopsy specimens of the liver were obtained, followed by complete autopsies.

Group III—One male and one female dog, numbers 5 and 6, were given daily injections of 1.66 mg. of alpha estradiol dipropionate in 1 c.c. of sesame

*The diethylstilbestrol dipropionate was supplied by the Department of Medical Research, Winthrop Chemical Company, New York. Estradiol was furnished by the Medical Research Division of the Schering Corporation, Bloomfield, New Jersey, and by Roche-Organon, Inc., Nutley, New Jersey.

oil. By the sixteenth day a marked thrombocytopenia had occurred. On the following morning the second biopsy of each liver was performed. After the biopsy an autopsy was done.

Group IV—Dogs 7 and 8, both females, were injected daily with 1 c.c. of sterile sesame oil for 16 days, and served as control animals for Group III.

Determinations of the percentage of glycogen in the liver were done by the method of Good, Kramer, and Somogyi.[11] A modification of Bloor's method described by Elman and Heifetz[12] was used for the determinations of fat. The wet liver sample was weighed, and the total nitrogen was measured by the method of Sobel, Yuska and Cohen.[13] Hematocrit determinations and measurement of serum albumin and globulin were obtained before and after each period of medication.

RESULTS

1. Chemical Changes.—In the table the results of the chemical analyses of the specimens of liver removed from the eight dogs prior to and after the administration either of the estrogenic substances or of the oily vehicles are presented.

2. Hematocrits and Blood Proteins.—The hematocrit and blood protein values obtained before and after the administration of the estrogens revealed no significant alterations.

3. Anatomical Changes.—The alterations in the gross appearance of the livers of the animals receiving the estrogenic substances were not striking. No distinction in the gross appearance of the livers could be made on the basis of the type of estrogenic substance administered. The site of the previous operation in each liver did not appear unusual.

For the microscopic examination each specimen of liver was divided into three contiguous samples. One was stained with eosin and methylene blue, another with Best's carmine and the third with scarlet red. By this method the amount and distribution of histologically demonstrable fat and glycogen were compared with the histologic picture in each specimen. In addition, comparisons of the histologic content and distribution of fat and glycogen and the histologic appearance were made among the different specimens.

LIVER BIOPSY SPECIMENS DURING CONTROL PERIOD

The microscopic appearance of the control specimens of liver removed surgically in the premedication periods was essentially normal. The eosin and methylene blue stain revealed a definite vesicular appearance in some of the hepatic cells. In most instances the hepatic cells of the central zone were less vesicular than those of the middle and in particular those of the peripheral zone. A comparison of the sections stained with eosin and methylene blue with sections from the same specimen stained with Best's carmine showed deposits of glycogen in the cells with a vesicular cytoplasm. Of the control specimens, the liver of Dog 1 appeared to contain the most and the liver of Dog 3 the least amount of glycogen. No sharp distinction could be made concerning the relative amount of glycogen present in the other six normal specimens of liver. The vesicular character of the cytoplasm of the hepatic cells as noted in the sections

CHEMICAL ANALYSES OF LIVER BIOPSY SPECIMENS FROM DOGS RECEIVING DIETHYLSTILBESTROL OR ESTRADIOL

DOG NO.	WT. KG.	MEDICATION	DAILY DOSE	NO. DAYS INJECTED	INTERVAL BETWEEN BIOPSIES —DAYS	LIVER ANALYSIS BEFORE (A) AND AFTER (P) INJECTIONS					
						GLYCOGEN %	DIFFERENCE %	FAT %	DIFFERENCE %	TOTAL NITROGEN %	DIFFERENCE %
Group I											
1	6.2	Diethyl stilbestrol dipropionate	5 mg.	17	21	(a) 6.28	−2.10	7.8	−4.2	2.50	+0.18
						(p) 4.18		3.6		2.68	
2	7.4	Diethyl stilbestrol dipropionate	5 mg.	17	21	(a) 5.34	+3.85	2.4	+0.1	2.87	−0.36
						(p) 9.19		2.5		2.51	
Group II											
3	5.6	Olive oil	1 c.c.	17	21	(a) 0.58	+3.19	5.2	−1.6	3.09	−0.10
						(p) 3.77		3.6		2.99	
4	6.6	Olive oil	1 c.c.	17	21	(a) 3.85	−1.07	3.2	−0.8	3.03	+0.09
						(p) 2.78		2.4		3.12	
Group III											
5	19.5	Alpha estradiol dipropionate	1.66 mg.	16	26	(a) 13.32	−10.9	1.3	+1.2	2.07	+0.78
						(p) 2.42		2.5		2.85	
6	15	Alpha estradiol dipropionate	1.66 mg.	16	26	(a) 4.85	−1.79	2.52	−0.02	2.83	+0.02
						(p) 3.06		2.5		2.85	
Group IV											
7	15	Sesame oil	1 c.c.	16	26	(a) 0.98	+3.32	3.9	−1.8	3.31	−0.50
						(p) 4.3		2.1		2.81	
8	17.5	Sesame oil	1 c.c.	16	26	(a) 1.1	+9.6	6.1	−4.7	3.23	−0.88
						(p) 10.7		1.4		2.35	

stained with eosin and methylene blue was not entirely due to the deposition of glycogen, since the tissue stained with scarlet red showed a minimal number of fine fat droplets in the cytoplasm of the hepatic cells. The droplets of fat tended to be larger and more numerous in the regions of the portal canals and the central veins. The control specimen of liver of Dog 1 and of Dog 3 appeared to contain the most fat. The other six control specimens contained a minimal amount of histologically demonstrable fat.

The microscopic appearance of the control specimen of liver removed surgically in the premedication period of each dog was compared with the microscopic appearance of the liver following the period of medication.

LIVER BIOPSY SPECIMENS AFTER DIETHYLSTILBESTROL

The two dogs in Group I received 5 mg. of diethylstilbestrol dipropionate daily for seventeen days. After the injections the liver of Dog 1 showed a slight increase in stainable glycogen and a moderate decrease in stainable fat. The decrease in fat was chiefly due to a decrease in the number of the larger fat droplets. There were rare vacuoles in the hepatic cells which failed to stain either as fat or glycogen. In the periportal spaces there was a slight increase in the number of mononuclear cells. After the diethylstilbestrol injections the liver of Dog 2 exhibited an appreciable increase in the content of stainable glycogen in practically all of the hepatic cells. There appeared to be a minimal increase in the amount of stainable fat in most of the parenchymal cells.

The two dogs in the control Group II received 1 c.c. of olive oil daily for seventeen days. After the administration of olive oil to Dog 3 the liver showed an appreciable increase in the amount of stainable glycogen and fat. In neither instance was the liver otherwise unusual.

LIVER BIOPSY SPECIMENS AFTER ESTRADIOL

The two dogs in Group III received 1.66 mg. of alpha estradiol dipropionate daily for sixteen days. The liver of Dog 5 exhibited a slight decrease in the stainable glycogen content after the estrogen injections. This decrease occurred in the middle and peripheral zones where glycogen was noted in the normal control specimen. The central zones were not appreciably involved either before or after the medication. The amount of stainable fat was scanty and no change could be discerned. Rarely vacuoles were noted which failed to stain either as glycogen or fat. After the administration of the estrogenic substance to Dog 6 the liver showed a minimal increase in the amount of stainable glycogen; however, the glycogen was confined chiefly to the cells of the peripheral and central zones. There was no alteration in the content of stainable fat.

The two dogs in control Group IV received injections of 1 c.c. of sesame oil daily for sixteen days. After the administration of sesame oil to Dog 7 the liver showed a moderate increase in the amount of stainable glycogen distributed rather uniformly throughout all zones of the hepatic lobules. No change was observed in the amount of stainable fat. After the sesame oil injections the liver of Dog 8 showed a definite increase in stainable glycogen. The glycogen was equally distributed throughout each lobule except for a somewhat smaller

amount immediately adjacent to the central veins. No change was noted in the content of stainable fat.

DISCUSSION

These observations reveal no consistent effect of estradiol or of diethylstilbestrol upon the livers of dogs. No correlation was established between administration of the estrogenic substances and chemical and histochemical changes in the liver.

A comparison of the values of hepatic glycogen obtained by chemical analysis before and after the administration of diethylstilbestrol dipropionate revealed a variable result. Following the injections there was a rise in the hepatic glycogen in one instance and a drop in another. Following the administration of alpha estradiol dipropionate there was a drop in the amount of hepatic glycogen in both examples; however, in one the drop was not appreciable. The hepatic glycogen in the control dogs before and after the administration either of sesame oil or of olive oil likewise showed a variation. It appears that the amount of glycogen in the liver may either increase or decrease following the administration of the estrogenic substances or of the oils alone.

A comparison of the values of the hepatic fat before and after the administration of the estrogenic substances showed inconsistent results. One of the two dogs receiving diethylstilbestrol showed a decrease in the amount of hepatic fat. The amount of fat in the liver of the other dog was unaltered. The two dogs injected with alpha estradiol dipropionate exhibited a rise in the hepatic fat in one instance and no change in the other. The four control dogs receiving either olive oil or sesame oil showed a variable but usually slight drop in the amount of hepatic fat. Thus, the administration of the estrogenic substances did not produce consistent changes in the amount of hepatic fat.

The total nitrogen content of the livers before and after the administration of either of the estrogenic substances or of the oils showed no appreciable change.

Investigators have reported evidence of hepatic damage in animals receiving large doses of estrogenic substances. In a previous paper two of us[5] described the occurrence of mild fatty degeneration and hydropic degeneration in the livers of dogs given equivalent doses of "natural" and synthetic estrogens. Furthermore, these livers contained a variable but usually scanty amount of glycogen as determined by Best's carmine stain. These observations were in disagreement with those of Teague,[6] who studied the effect of diethylstilbestrol and of estradiol on the livers of rats. He concluded that the vacuolization of the hepatic cells was due to an accumulation of glycogen and not to fatty degeneration. Recently Elman and Heifetz[12] demonstrated extensive vacuolization of the parenchymal cells of the liver in dogs maintained upon a protein deficient diet. Chemical analyses and stained sections of the livers showed the vacuolization not to be due to the accumulation of fat or of glycogen. In the present study, in order to determine the nature of the microscopic changes in the liver, concomitant chemical and histochemical analyses were done. The results of the chemical analyses showed little change in the value of the total hepatic nitrogen, after the administration of the estrogenic substances. A histologic comparison rarely showed a vacuole in the hepatic cells which failed to

stain either for fat or for glycogen. On the basis of the work reported by Elman and Heifetz it does not seem probable that the hepatic vacuoles in the present study were due to a deficiency of protein. In the previous study[5] of the effect of estrogens upon the liver, vacuoles were encountered which did not stain either with scarlet red or with Best's carmine. However, the dogs received larger amounts of estrogenic substance over a longer period of time than did the dogs in the present investigation, and death was often preceded by a period of cachexia.

In the present study there was no exact correlation between the quantitative chemical content of glycogen in the liver and the amount as determined by histochemical estimation. The lowest value of hepatic glycogen determined by chemical analysis occurred in a liver which also appeared to have the least glycogen by histochemical estimation. It was rarely possible to estimate correctly the amount of glycogen in the liver by histochemical means in that group of livers which contained the most glycogen. This conclusion is essentially in agreement with the observations of Grafflin, Marble, and Smith.[14] These authors made histologic estimations of the content of glycogen in the livers of guinea pigs and compared the result with the chemically determined glycogen content of each liver. They concluded that histologic estimation of the hepatic glycogen was at best only approximate. In the present study there was some variation in the occurrence of the glycogen in different areas of the same sample of tissue. This fact may in part explain the unreliability of the histologic estimations of hepatic glycogen.

A comparison of the histologic estimation of the hepatic fat with the amount present as determined by quantitative analysis shows only approximate correlation. Those specimens of liver in which fat appeared more abundant by histologic estimation generally showed larger amounts by quantitative analysis. When the amount of fat was low, as determined chemically, the histologic estimation frequently showed little correlation with the results of the chemical analysis.

Janes and Nelson[15] and Griffiths, Marks and Young[16] have demonstrated by chemical analysis an increase of the hepatic glycogen following the administration of estrogenic substances to rats. This result is in accord with Teague's observation of an histologic increase of hepatic glycogen in the rat following estrogenic medication. The results of these workers are at variance with observations in the present study on dogs. It is known that estrogens may produce certain blood changes in one species of animal, but little or no corresponding effects in other species. It is possible that the metabolism of the liver is also affected differently in various species of animals following the administration of estrogenic substances. The results of this study indicate that in dogs neither hepatic glycogen nor hepatic fat are consistently altered by the amounts of estrogens and the durations of administration used in these experiments.

SUMMARY AND CONCLUSIONS

1. Hepatic glycogen, fat and nitrogen as determined by chemical analysis of biopsy specimens showed no significant alteration following the administration to dogs of diethylstilbestrol dipropionate or of alpha estradiol dipropionate.

2. The histologic estimation of the amount of hepatic glycogen and hepatic fat could be only approximately correlated with the chemical analyses.

3. Morphologic changes such as hydropic or fatty degeneration of the liver, which we have previously found in dogs receiving large doses of synthetic or natural estrogens, were not observed in this study. This may be explained by the absence of the profound blood changes previously induced.

The authors express appreciation to Mrs. Harriet Wolf Davey and to Miss Ellabeth Houghton for valuable technical assistance.

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EFFECTS OF ETHER ANESTHESIA UPON TOTAL ERYTHROCYTE AND WHITE CELL COUNTS OF ADULT FEMALE RATS

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THE use of anesthesia in hematologic investigation has long been discouraged. Hemodilution, hemoconcentration, and contraction of the spleen have been described in texts of anesthesiology and pharmacology with the use of anesthesias of various types. Several recent papers have described a hemoconcentration in dogs with the use of ether anesthesia.[1-5] Barbour,[6] however, found no hemoconcentration with ether in two rabbits, and Conley[7] and Jarcho[8] reported no hemoconcentration with ether anesthesia in cats. Creskoff, Fitz-Hugh, and Farris[9] reported that light ether anesthesia could be used in the rat without significantly altering the total erythrocyte count, though these authors do not quote any evidence for this statement. From the above data it seems that all species do not respond to ether anesthesia in a similar manner as far as plasma volume and the contraction of the spleen are concerned.

The present work on the effects of ether anesthesia upon the number of formed elements found in the blood of adult female rats was undertaken because (1) no detailed study has been reported on the effect of ether anesthesia on the total erythrocyte or white cell counts of the rat, and (2) since this animal is being used for the study of possible effects of endocrines upon the formed elements of the blood, it would be convenient to use a light ether anesthesia in obtaining blood samples.

METHODS

Adult female rats of the Long-Evans strain were employed for this study. They were 3 to 4 months of age and weighed 100 to 150 grams.

Blood was obtained by heart puncture, 0.1 c.c. of blood being removed for each sample. The blood was placed in a small vial containing a minute amount of powdered heparin to prevent clotting. Total erythrocyte and white cell counts were made with standard pipettes and an improved Levi-Hausser counting chamber. The same individual made all the counts.

RESULTS

In the first part of this study, 21 adult female rats were studied in an attempt to determine if light ether anesthesia for short periods of time alters the total erythrocyte or white blood cell counts (Table I). Each rat was lightly anesthetized and blood obtained by heart puncture. Less than two minutes elapsed between the time the rat was removed from the ether jar and the blood was obtained, the average time usually being one minute. This procedure, using ether anesthesia, was repeated three times at 10-day intervals. Total

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Received for publication, May 28, 1944.

erythrocyte and total white blood cell counts were done on the 63 samples of blood. The average total erythrocyte count was 8.38 million cells per cubic millimeter, with a mean deviation* of 0.46 million cells. The total white count was 7.92 thousand cells per cubic millimeter, with a mean deviation of 1.75 thousand cells. These figures compare favorably with a previous study of 62 unanesthetized adult female rats of this same strain[10] in which the total erythrocyte count was 8.26 (±0.68) million cells per cubic millimeter and the total white count was 8.88 (±2.87) thousand cells per cubic millimeter.

Three more blood samples were then taken at 10-day intervals from each of these same 21 adult female rats using no anesthesia whatever, the same rats being used to eliminate individual differences. The average of the total erythrocyte counts of these 63 samples of blood was 8.39 million cells per cubic millimeter, with a mean deviation of 0.59 million cells; the white cell count 6.67 thousand cells per cubic millimeter, with a mean deviation of 1.65 thousand cells.

TABLE I

EFFECT OF LIGHT ETHER ANESTHESIA ON TOTAL ERYTHROCYTE AND WHITE CELL COUNTS OF ADULT FEMALE RATS

	LIGHT ETHER ANESTHESIA	NO ANESTHESIA
Rbc in millions	8.38 ± 0.46*	8.39 ± 0.59
Wbc in thousands	7.92 ± 1.75	6.67 ± 1.65

*Mean deviation.

These data indicate that the total erythrocyte count of the rat is not significantly altered by a short period of ether anesthesia. The total white cell count showed a rise from 6.67 thousand cells with no anesthesia to 7.92 thousand cells with anesthesia. This difference is not a significant one. The total white cell counts are quite variable under the best of conditions. Both of these average figures are within the normal range for total white cell counts in this strain of rats. The above data are summarized in Table I.

As all previous studies by other workers on the effects of ether anesthesia on the plasma volume and the contraction of the spleen utilized the ether for prolonged periods of time rather than one to two minutes, it was thought that a study of the effects of a longer period of ether anesthesia on the rat would be of interest. Accordingly, in the second part of this work, 10 adult female rats were anesthetized with ether and 0.1 c.c. of blood removed by heart puncture at 2, 6, 10, 20, 30, and 60-minute intervals after the rat was removed from the ether jar. Total erythrocyte and white cell counts were done on each sample of blood (Table II).

The average erythrocyte count for the 2-minute interval was 8.37 million cells per cubic millimeter. This agrees with the figures of 8.38 and 8.39 million cells obtained in the first part of this work. The total erythrocyte averages of 8.33, 8.20, 8.02, 7.96, and 7.84 million cells for the 6, 10, 20, 30, and 60-minute intervals respectively show a gradual decrease in the total number of erythrocytes per cubic millimeter. This decrease has been tested with various tests of

*As obtained by the formula: $E = \sqrt{\frac{\Sigma d^2}{N-1}}$

TABLE II

EFFECT OF PROLONGED ETHER ANESTHESIA ON TOTAL ERYTHROCYTE AND WHITE CELL COUNTS OF ADULT FEMALE RATS

	PERIOD OF ETHER ANESTHESIA IN MINUTES					
	2	6	10	20	30	60
Rbc in millions	8.37 ±0.51*	8.33 ±0.83	8.20 ±0.55	8.02 ±0.57	7.96 ±0.70	7.84 ±0.67
Wbc in thousands	6.45 ±1.60	7.32 ±2.11	6.41 ±1.45	6.42 ±2.05	6.64 ±1.30	6.94 ±2.40

*Mean deviation.

significance. It is not a significant change. The figure 7.84 million cells per cubic millimeter is within the standard deviation and any significant change should be at least twice the standard deviation. In addition, the above change is not biologically significant as many normal adult female rats show a count of 7.80 million cells per cubic millimeter. The total white count showed no significant change.

These data indicate that prolonged use of ether anesthesia, at least for sixty minutes, does not materially alter the total erythrocyte or total white cell counts in adult female rats (Table II).

As the time factor seemed to produce no pronounced effects, it was of interest to determine if the depth of anesthesia was the important factor. Fourteen adult female rats were very lightly anesthetized for fifteen minutes. Blood samples were then taken by heart puncture. After a 10-day interval the same rats were deeply anesthetized for the same length of time, fifteen minutes. Blood samples were again obtained by heart puncture and total erythrocyte counts made on each sample (Table III).

The total erythrocyte values of 7.84 million cells per cubic millimeter for the lightly anesthetized group, and 8.11 million cells for the deeply anesthetized group are both normal values. These data indicate that neither light nor deep ether anesthesia alters the total erythrocyte count of adult female rats.

TABLE III

EFFECTS OF LIGHT AND DEEP ETHER ANESTHESIA ON TOTAL ERYTHROCYTE COUNT OF ADULT FEMALE RATS

	LIGHT ETHER ANESTHESIA FOR 15 MINUTES	DEEP ETHER ANESTHESIA FOR 15 MINUTES
Rbc in millions	7.84 ± 0.80*	8.11 ± 0.64

*Mean deviation.

DISCUSSION

Ether anesthesia does not produce changes in the blood concentration in all animals. Although a hemoconcentration follows ether anesthesia in the dog, the rabbit and cat do not demonstrate any such change in blood concentration with the same anesthesia. The rat appears to belong in the group with the rabbit and the cat. The Long-Evans rats used in this study showed no significant change in the total erythrocyte or white cell counts when under the influence of ether anesthesia. Light anesthesia, deep anesthesia, anesthesia for short periods of time, and anesthesia for long periods of time produced no significant changes.

The source of this blood, the heart, should be emphasized. Whether peripheral blood would show any different results is problematical.

These data would seem to indicate that a light ether anesthesia for a 1- to 2-minute interval will in no way alter the number of formed elements in the blood of Long-Evans adult female rats when the heart is used as a source of blood. If anesthesia is used, heart punctures can be done repeatedly on rats with no signs of injury. The heart puncture following a short period of ether anesthesia will be utilized as a method of obtaining blood from rats in subsequent work in this laboratory.

SUMMARY

This work was an attempt to determine if ether anesthesia would alter the number of the formed elements in the blood of mature rats. Adult female rats of the Long-Evans strain were anesthetized for a 1- to 2-minute period, for a 60-minute period, anesthetized lightly, and anesthetized deeply. There was no significant change in either the total erythrocyte or the total white cell counts. Blood was obtained from the heart.

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A VASOSPASTIC FACTOR IN THE SERUM OF A CASE OF RAYNAUD'S DISEASE WITH COLD AGGLUTINATION. EXPERIMENTS ON RABBITS

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THE case (Mrs. R.)† with which these experiments are concerned has already been published on account of the association of "Cold autohemagglutination with Raynaud's Disease" (Benians and Feasby, 1941). The relation of this condition to hemoglobinuria e frigore, in which Raynaud's syndrome is frequent, was pointed out and it was suggested that the vascular spasm might be allergic, i.e., an antigen-antibody stimulus leading to a vascular spasm, in this case a reversible process governed by the temperature of the blood. There are numerous substances which injected intravenously cause anaphylactoid death in the rabbit, and among these are cyto-toxic sera which are rich in heterophile antibodies. The serum of Mrs. R. contains agglutinins and lysins which act on rabbit's blood cells both in the cold and at body heat. The action of the antibodies on the rabbit blood cells is apparently not in itself the cause of the rapid anaphylactoid death which is due to spasm of the pulmonary arteries. It might, of course, be the case that this serum contains a toxic body which causes an arterial spasm having no relation to antigen-antibody reactions, but a mechanism of allergic type seems inherently probable. A few experiments in prophylactic treatment based on the idea of immunity reactions have been carried out and are recorded later.

Series 1.—Effects of "normal" serum. Rabbits are regarded as being exceptionally sensitive to intravenous injections of protein, so that some direct comparison is useful. Animals of various sizes as available were injected intravenously in a single injection with various amounts of "normal" human serum. Of these fourteen animals one died an anaphylactoid death, a second died a few hours after injection and probably as a result of it. Two were definitely shocked in the sense that they lay down and passed urine and feces but recovered after about fifteen minutes. The other ten suffered no obvious ill effect although three of them received 4 c.c. or more. Some of these sera taken at random from the laboratory bench evidently contained a toxic factor, the six rabbits, however, which were injected with serum from healthy "donors" showed no reaction at all, and this is in striking contrast with the fatal effects obtained with much smaller doses of the serum (Mrs. R.).

With the limited number of rabbits available one cannot be said to have fixed a minimal lethal dose but from Table II, and controls to individual experiments

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†Under the care of Dr. R. Kempthorne, North Middlesex County Hospital.

Received for publication, Feb. 10, 1944.

TABLE I

WEIGHT OF ANIMAL	AMOUNT OF SERUM	SOURCE	RESULT
2.28 Kg.	4 c.c.	Donor*	Nil
2.28 Kg. (Same rabbit 2 days later)	4½ c.c.	Donor	Nil
1.14 Kg.	2 c.c.	Pooled sera from 2 W.R. cases	Nil
1.14 Kg.	3 c.c.	Pooled sera from 4 W.R. cases	Anaphylactoid death in 5 minutes
1.82 Kg.	2 c.c.	Pooled W.R. serum	Nil
0.79 Kg.	2 c.c.	Pooled sera	No effect that day but found dead next day. No P.M.
1.14 Kg.	2 c.c.	Pooled sera	Nil
1.14 Kg.	3 c.c.	Pooled sera	Nil
1.59 Kg.	3 c.c.	Pooled sera	Uneasy ¼ hour then recovered
2.28 Kg.	4½ c.c.	Donor	Nil
1.14 Kg.	2 c.c.	Donor	Nil
2.28 Kg.	2 c.c.	Old standing anemia now much improved	Uneasy ¼ hour then recovered
1.36 Kg.	4 c.c.	Pooled sera from 3 donors	Nil
2.0 Kg.	3 c.c. plasma	Donor	Nil

*Different "donors" used on each occasion. Serum in all cases given intravenously at ordinary laboratory temperature.

TABLE II

EFFECTS OF SERUM (MRS. R.) GIVEN INTRAVENOUSLY AT LABORATORY TEMPERATURE

WEIGHT OF ANIMAL	DOSE	EFFECT
2.28 Kg.	5 c.c. fresh serum	Death in 1 minute
2.96 Kg.	5 c.c. fresh serum	Death in 1 minute
1.82 Kg.	1.5 c.c. fresh serum	Death in 1 minute
2.65 Kg.	1 c.c. fresh serum	Death in 9 minutes
2.88 Kg.	1 c.c. fresh serum	Moderate shock but survived
1.6 Kg.	1 c.c. fresh serum	Death in 5 minutes
1.0 Kg.	1 c.c. fresh serum	Death in 2½ minutes
1.0 Kg.	1 c.c. fresh serum	Death in 2 minutes

TABLE III

COMPARISON OF EFFECTS OF THE SAME SAMPLES OF SERUM (MRS. R.) GIVEN AT LABORATORY TEMPERATURE AND WARMED TO 37° C.

1.59 Kg.	2.0 c.c.	10-day-old serum	Death in 10 minutes
1.48 Kg.	2.0 c.c.	Same serum warmed to 37° C. for 5 minutes and given at 37° C.	Shocked but recovered
2.28 Kg.	2.5 c.c.	6-day-old serum	Death in 2 minutes
2.28 Kg.	2.5 c.c.	Same serum warmed to 37° C. for 1 hour and given at 37° C.	Shocked but recovered
1.59 Kg.	1.0 c.c.	Fresh serum	Death in 2½ minutes
1.58 Kg.	1.0 c.c.	Same serum warmed to 37° C. for 15 minutes and given at 37° C.	Shocked but recovered
1.5 Kg.	0.5 c.c.	Fresh serum	Death in 5 minutes
1.45 Kg.	1.0 c.c.	Same serum warmed to 37° C. for 30 minutes and given at 37° C.	Severe shock for 1 hour but recovered
1.8 Kg.	1.0 c.c.	Fresh serum warmed to 37° C. for 30 minutes and given at 37° C.	Very severe shock; gradual recovery
2.3 Kg.	2.0 c.c.	Fresh serum warmed to 37° C. for 1 hour and given at 37° C.	Death in 5 minutes

given later, it appears probable that 1 c.c. of the fresh serum has a fatal effect on rabbits up to about 1.5 to 2 Kg. in weight.

In the experiments to be described later where a control test was carried out concurrently it is bracketed with its partner.

It is to be noted that although the warmed serum in equal or larger amount than the unwarmed has lost its lethal effect, except in one instance, it can still produce a considerable degree of shock. In the state here described as shock the animal lies down and passes urine and often feces, the ears are pale and cold, breathing is rapid, and the animal is limp to handle. There is no evidence of pain or paralysis. If put on its legs it will move off slowly but naturally to another part of the room. Recovery is gradual, usually in about half to one hour.

TABLE IV

A FEW TENTATIVE EXPERIMENTS SHOWING THE EFFECT OF TREATING THE FRESH SERUM (MRS. R.) IN VARIOUS WAYS

WEIGHT OF ANIMAL	DOSE OF SERUM INTRAVENOUSLY	EFFECT
(1) *Heated to 56° C. for 30 minutes*		
1.5 Kg.	2.0 c.c.	Shocked but recovered
2.3 Kg.	3.0 c.c.	Slight shock only
1.5 Kg.	1.5 c.c.	No effect
(2) *Extracted by shaking with powdered activated charcoal, stood 30 minutes at laboratory temperature. Centrifuged clear*		
0.9 Kg.	2.0 c.c. extracted	Nil
1.6 Kg. (control)	1.0 c.c. untreated	Death in 5 minutes
1.5 Kg.	2.0 c.c. extracted	Nil
2.3 Kg.	3.0 c.c. extracted	Nil
2.5 Kg.	3.0 c.c. extracted	Nil
(3) *Extraction with washed packed human Group O corpuscles in equal bulk, shaken and kept at 4° C. 90 minutes then centrifuged clear in a cold bucket (marked hemolysis)*		
2 Kg.	3.0 c.c.	Nil
(4) *Globulins salted out from 5 c.c. dialysed and redissolved in 5 c.c. of saline*		
2.5 Kg.	5.0 c.c.	Death in 2 minutes
(5) *Effect of storage in refrigerator*		
1.7 Kg.	2.0 c.c. 5 weeks old sterile serum	Moderate shock, recovery
1.7 Kg. (control)	1.0 c.c. fresh serum	Death in 4 minutes

The experiments so far show definitely that the serum (Mrs. R.) contains a highly toxic factor for rabbits; the diminution of the lethal power of the serum when given warmed to 37° C. makes it almost certain that this factor is a "cold antibody."

Post-mortem examinations were carried out immediately after death on many of the cases and showed the general characters of anaphylactoid death in rabbits. No detailed study of the organs was made. Rapidly fatal cases usually showed as follows. Left heart tightly contracted and almost empty. Right heart and systemic veins, especially those of the abdomen and portal system, heavily distended with dark blood. A little clear free fluid in the abdomen in some cases. Pleurae clear. Lungs pale, limp and shrunken, only bleeding slightly at the cut surface. Stomach and intestine deeply congested. Kidneys

deep plum color on surface, on section the deeper cortical zone is relatively pale. Suprarenals show no abnormality. The blood shows some lysis, but agglutination was not a marked phenomenon. Coagulation of the blood in some fatal cases was delayed for upward of 1½ hours in vitro, the clot was very fragile.

Microscopic sections of a few organs as follows. Cardiac septum anteriorly—coronary vessels empty but not contracted. Lung near hilum—tight contraction of the large arteries, most of which are nearly closed and empty, ruptures of the vessel walls are seen and many arteries lie in a wide sheath of extruded blood limited by the distended adventitial coat. The muscle fibers of the arteries in places show a very marked vacuolation with what appears to be complete destruction. Hemorrhage into alveoli was not noted to any considerable degree. There was some emphysema. Kidneys—the complex vascular mechanism has not been fully examined but in some cases marked contraction of the arcuate arteries is seen with severe vacuolation of muscle fibers of the media. This vacuolation is very much more marked than that seen in control rabbits killed by other methods, including a blow on the neck, coal gas, and chloroform, though it cannot be asserted that shock is entirely excluded in any of these forms of death. The cause of death is beyond doubt pulmonary artery spasm with obstruction to the blood flow.

PROTECTION AGAINST THE SERUM BY INJECTION OF LIPOID

Bearing in mind the relation of lipoids to the cell membrane in respect of permeability, their power of adsorption, the role of lecithin as hapten of the Forssman antigen, and especially its ability to function in the Wassermann reaction, it was decided to try its effect empirically as a protective agent. In the small number of experiments below it is shown to have had a protective effect in most instances. The suspensions of lecithin were made by floating a saturated alcoholic solution of ovolecithin on saline and gently rolling the tube to a uniform turbidity. Proportions of 10 per cent and 20 per cent of the alco-

TABLE V

WEIGHT OF ANIMAL	LECITHIN EMULSION	SERUM (MRS. R.)	EFFECT
2.3 Kg.	4.0 c.c. (10%)		No harmful effect
2.3 Kg.	4.0 c.c. (20%)		No harmful effect
2.7 Kg.	8.0 c.c. (20%)		No harmful effect
1.7 Kg.	7.0 c.c. (20%)		No harmful effect
1.75 Kg.	3.5 c.c. (10%)	2.5 c.c. (10-day-old serum	No harmful effect
2.3 Kg.	5.0 c.c. (20%)	2.5 c.c. fresh serum	Shocked for ½ hr. partial recovery; died following night
1.75 Kg.	5.0 c.c. (20%)	1.0 c.c. fresh serum	No effect
1.75 Kg. (control)	-	1.0 c.c. fresh serum	Death 2½ minutes
1.6 Kg.	5.0 c.c. (10%)	1.0 c.c. fresh serum	Slight shock, recovery
1.6 Kg.	5.0 c.c. (10%)	1.0 c.c. fresh serum	Slight shock, recovery
1.6 Kg. (control)	-	0.5 c.c. fresh serum	Death 5 minutes
1.5 Kg.	4.0 c.c. (20%)	1.0 c.c. fresh serum	Death 5 minutes
1.5 Kg. (control)	-	0.5 c.c. fresh serum	Death 6 minutes
0.9 Kg.	4.0 c.c. (20%)	1.0 c.c. fresh serum	Death 4 minutes
1.0 Wg. (control)	-	0.5 c.c. fresh serum	Death 4 minutes

holic solution were used in experiments and the lecithin was injected intravenously just before injection of the serum.

The effect of lecithin itself was tested in a few preliminary experiments. The alcohol factor was not controlled. All injections were made at ordinary laboratory temperature. The lecithin used in most of the experiments was a pre-war jar of commercial ovolecithin (British drug houses) which had gone a deep brown color. A fresh pale preparation was used in a few of the experiments. Unfortunately the actual preparation used in any individual experiment was not recorded.

DISCUSSION

It may be that these experiments do not actually prove the point at issue which is that arterial spasm results directly from the action of "cold antibodies." They raise, however, a number of speculations which can be linked together.

The experiments relate to the serum of a patient which contains cold panhemagglutinins in high titer, and some lysins. This patient suffers from Raynaud's syndrome when cooled and, when really cold, exhibits a condition that may be called shock, with a feeling of constriction in the chest, shortness of breath, pale pinched facies, and blueness of the extremities. Her serum injected cold into rabbits causes lysis with a little agglutination of the blood and rapid death due to spasm of arteries. The spasm affects mainly the pulmonary arteries which may be ruptured, but is also seen in some of the renal arteries and in the vessels of the ears. The serum when given at body heat is seen to have lost much of its toxicity.

It seems likely that this violent effect in the rabbit has its counterpart in a mild and reversible form in the patient and that both could be regarded as allergic phenomena. Admitting that the experimental animal and the clinical case are widely different entities they have, in the experiments described, the essential point in common, i.e., that each shows arterial spasm when a "cold" panagglutinating antibody is circulating in the blood stream.

Antigen or antibody introduced into the blood stream and finding a suitable partner in the fixed tissues may unite with it and produce a reaction known as allergic or anaphylactic shock. Surface action, that is to say agglutination, precipitation, etc., is the obvious feature of antigen-antibody combination and the intense vacuolation seen in the muscle fibers of the media of the rabbit's pulmonary arteries bears some comparison with the dramatic clot-like agglutination which this serum causes in vitro when mixed with whole blood in the cold, and suggests an equally direct action on the muscle itself. It should be said that authorities, Topley and Wilson (1936) quoting various authors, regard the type of reaction shown in these injected rabbits as anaphylactoid and due to cytotoxic bodies in the serum, and this would be a feasible alternative explanation. For present purposes it is not necessary to adopt any specific nomenclature, provided the effects of this serum can be interpreted in terms of surface action.

There is some clinical support for a hypothesis that the action of autoantibodies is related to vascular spasm. In a review of the hemoglobinurias Witts (1936) says "a feature that has not attracted the attention it deserves

is the occurrence of vascular disturbances which may threaten the life and comfort of the patient almost as seriously as the anemia or the suppression of urine." He also points out that shock is a prominent feature in intravascular hemolysis. Stats and Bullowa (1943) in an examination of all published cases of cold agglutination find twelve of them (roughly perhaps 30 per cent) showing functional vascular disease of the extremities. Their own case is one of only two in which the process has gone on to gangrene.

However attractive an allergic hypothesis might seem to be it is clear that the mere presence of high titer cold agglutinins does not necessarily lead to vasospasm. This effect so far seems to be limited to a class of case in which intravascular lysis is also taking place. An exception is a case of acrocyanosis recently reported by Helwig and Freis (1943) following atypical primary pneumonia; in this case there was no hemolysis and the authors attribute the stasis to the agglutination of the red cells. Stats and Bullowa had already adopted the same explanation in their case despite the fact that the obstruction was irreversible on warming.

Autoantibodies certainly exist in the blood plasma or they may develop or increase as the result of disease or experimental processes, thus Karady (1939) has apparently produced allergic shock in guinea pigs by exposure to both heat and cold, and seeks to explain some human allergies on this basis.

In the blood the iso-antibodies are always compatible with their accompanying cells, but in the "cold" antibodies we are concerned with factors which apparently are always incompatible, given adverse physiological conditions. Agglutinins and lysins of this type are known and the action of both is well seen in hemoglobinuria e frigore. It has to be borne in mind that, as ordinary immune bodies, these two factors are closely related and up to a point may be identical.

Cold hemagglutinins first demonstrated by Landsteiner (1903) are apparently a normal component of the plasma and at temperatures below 5° C. may be demonstrated in small amounts in most sera. When they are pathologically increased they usually function at higher temperatures, up to 25° C., although their power falls off rapidly as the temperature rises. Rosenthal and Corten (1937) in a study of these bodies conclude that the high titer cold agglutinins are not derived from an increase of normal "cold" antibodies for two reasons (1) that they can function at a higher temperature, and (2) that the reaction is massive and sudden. The point cannot be argued here but it seems probable that these are features of the strength of the factor rather than of its nature. It must be considered at present an open question whether the normal cold agglutinin is related to that which occurs during infections such as trypanosomiasis and atypical primary pneumonia, or whether either is the same as that which occurs in cold hemoglobinuria or, on rare occasions, in the hemolytic anemias.

Putting on one side the normal cold agglutinins it will be of interest to consider broadly and in two groups the bulk of the diseases in which a high titer serum is commonly found, because they appear at first sight to have no sort of connection with each other. The first group would include trypanosomiasis and atypical primary pneumonia. The former is essentially a protozoal

infection of the blood stream and in many aspects its morbid anatomy is akin to syphilis. As well as cold agglutinins a pseudopositive Wassermann reaction occurs in this disease. The latter disease, though not yet well defined, is believed to be due to a virus infection. In this condition Drew, et al. (1943) state that a positive Wassermann reaction is not uncommon. Cold agglutinins were demonstrated by Peterson (1943) and used as a diagnostic measure by Turner, et al. (1943) who have shown that these agglutinins show a rise and fall in the manner of other immune bodies.

The second group includes cold hemoglobinuria, in which Raynaud's syndrome is a frequent and severe symptom and cold agglutinins and lysins are constant; and secondly those cases of cold agglutination associated with hemolytic anemia or some other form of blood disease. In the first of these (cold hemoglobinuria) a positive Wassermann reaction is so frequently present as to lead to its being regarded as a syphilitic disease, which of course it may be, although a diagnosis of syphilis based only on the Wassermann reaction may be fallacious in such cases owing to the presence of high titer heterophilic antibodies in the serum. In the hemolytic anemia type of case a positive Wassermann reaction is also frequent. Apart from the cold agglutinins the Wassermann body thus forms a tenuous link binding these diverse diseases together.

The Wassermann body is regarded as a product of autoimmunization to damaged tissues and, since syphilis is essentially a vascular disease, it is not a mere coincidence that the other factor functioning in the complement fixation test should be an extract of vascular-system tissue, i.e., heart muscle. It is to be noted that lecithin can also function in this reaction as an alternative to heart muscle.

We have then some reason for regarding the high titer cold agglutinin as an immune body arising in many instances parallel with the Wassermann body and perhaps mainly as a reaction to blood vessel damage. In its turn it could, theoretically, affect the media of the arteries (for this is the tissue mainly damaged in syphilis) and so lead to spasm, although admittedly the Wassermann body does not in general react so itself. The easiest explanation of why its spastic action is mainly seen in the cold would be to presume that it was built up on an autochthonous prototype. Turner, et al., while taking many of the above-mentioned factors into consideration conclude that the cold agglutinin arising in atypical primary pneumonia may be either a direct antibody to the unknown infecting agent, or a response to damaged tissues perhaps of the respiratory tract.

Seeing that cold agglutinins are a normal constituent of the blood plasma it might not be out of place to consider finally whether they have, or have not, a teleological value to the organism. Their function, if any, would lie in stopping the blood flow in an excessively cooled part at the periphery and so preventing further loss of heat in the blood still circulating. Vasomotor control through the nervous system is a relatively late arrival in the course of evolution and it may be that some controlling mechanism of humoral type preceded it.

The first response of superficial vessels to cold is a local one as Lewis (1941) has shown and the contraction affects vessels of all types, but no doubt

the arterial types most, on account of their muscular element. It is in this mechanism of contraction that a physiologic factor in the cooled plasma might come into play. This, however, is a mere supposition, in any case it is probably a truism (altering Osler's dictum) that man is as cold as his arteries.

SUMMARY

A serum containing high titer cold antibodies, and derived from a case of Raynaud's disease, is shown to cause fatal pulmonary artery spasm in rabbits when given, cold, intravenously. This effect is mitigated by giving the serum warm. It is suggested that these cold antibodies have a direct effect, probably of an allergic type, on arterial musculature both in the experimental animal and the clinical case. It is further suggested that the frequent association of cold antibodies with a Wassermann-like body point to an origin of the former from diseased vascular structures and this again would help to explain their action on both blood cells and vessels. A possible function of cold antibodies in normal vascular control is mentioned, these may or may not be the basis or protoype of the high titer cold antibodies. Preliminary experiments in protection against the cold antibodies by the intravenous injection of lipoids have been carried out with some success.

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THE COLD-SUSCEPTIBLE GLOBULIN FRACTION OF PATHOLOGIC SERA

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IN AN earlier paper it was shown that sera of dogs and human beings infected with kala-azar contain a protein fraction closely related to, but even more labile than, euglobulin.[3] An outstanding characteristic of this pathologic fraction is its deposition as a flocculent precipitate in the cold and its tendency to revert to a dissolved state when the temperature is suitably raised (37° C.). It was designated the Cold Fraction (C.F.) and defined as that protein portion of serum which precipitates when the serum stands for twenty-four hours at from 7 to 11° C. The present investigation deals further with the significance of the C.F.

METHODS

Findings reported in our earlier paper formed the basis of the present. It was found necessary in many cases to determine the amount of the C.F. after three days rather than after twenty-four hours of standing in the refrigerator, since precipitation in the shorter interval was often incomplete. A slight serum turbidity is often observed in pathologic cases. It has not been connected diagnostically with any particular condition. Successive grades of abundance of C.F. in serum were designated as follows: 1, turbidity marked but no precipitate; 2, light precipitate; 3, flocculent precipitate; 4, marked precipitate easily estimable by quantitative methods; 5, precipitate of a different nature present as a layer on the surface.

Essential features of the reversion of precipitation have been described in an earlier paper. Chemical methods were as described in our earlier paper.

Dilution fraction (D.F.) designates that fraction of globulin which is particularly sensitive to absence of electrolytes and which precipitates five minutes after dilution of 1 part serum with 30 parts of distilled water.

RESULTS

C.F. in Human Beings Infected With Kala-Azar.—Five patients with kala-azar were examined. Two had mild infections and could be tested on one occasion only. In both of these C.F. grade 3 was recorded. The results obtained in the three other patients with kala-azar will be reported in detail because they are in certain respects peculiar (Table I).

Case S. represents an infection with kala-azar derived from Turkestan. The disease at examination was less than of five weeks' duration. The condition was relatively mild and readily amenable to treatment. It is noteworthy that although the formol-gel reaction was found negative, the D.F. only a trace, and

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TABLE I

HUMAN BEINGS WITH KALA-AZAR

PATIENT	DATE	TOTAL PROTEIN	ALBUMIN	GLOBULIN	EUGLOBULIN	C.F.	D.F.	FORMOL-GEL	REMARKS
S.	6/10/43	8.36	3.78	4.58	Slight increase	1.56%	Trace	Ø	Treated with stilbene
	6/21/43	6.70	3.60	3.10	0.29	3	Trace	Ø	Treated with stilbene
	7/ 6/43	7.50	4.42	3.08	Slight increase	Trace	Ø	Ø	Clinical improvement
	8/11/43	6.65	4.70	1.95	Normal	Ø	Ø	Ø	Clinically normal
P.	6/ 7/42	10.00	–	–	4.20	3	+	Instantaneous +	Treated with stilbene
	7/ 2/42	10.80	2.88	7.92	4.40	3	1.86%	Instantaneous +	Treated with stilbene
	7/31/42	13.00	1.30	11.7	1.25	3	2.37%	Instantaneous +	Treated with stilbene
	8/ 6/42	10.80	–	–	Increase	0.2%	+	Instantaneous +	Treated with stilbene
	8/13/42	10.44	3.24	7.20	4.31	0.22%	1.16%	Instantaneous +	Clinically normal
	9/ 7/42	8.52	4.44	4.08	Heavy increase	0.24%	+++	Instantaneous +	Clinically normal
	10/11/42	10.05	3.93	6.12	Increase	0.30%	++	Instantaneous +	Clinically normal
	1/21/43	8.72	5.50	3.22	Normal	3	++	+ (After several hours)	Clinically normal
G.	2/ 3/42	9.23	2.70	6.53	Heavy increase	3-4	++	+ (After 2 hr.)	
	2/24/42	8.04	3.43	4.61	2.48	3-4	+	+ (After 3½ hr.)	
	4/14/42	7.18	3.44	3.74	Increase	3-4	+	+ (After 6 hr.)	
	5/ 1/42	7.72	3.95	3.77	Increase	3-4	(+)	+ (After 2 hr.)	
	7/30/42	6.09	4.21	1.88	Slight increase	3	Trace	Ø	Clinically normal
	9/25/42	5.71	3.24	2.47	Normal	3	Ø	Ø	Clinically normal
	3/ 1/43	7.44	4.90	2.54	Slight increase	3	Trace	Ø	Clinically normal

euglobulin increased but slightly, the C.F. was highly abundant. It disappeared rapidly together with the recession of the total globulin under treatment. Whether, as this suggests, presence of C.F. is of diagnostic value in early stages of kala-azar can be decided only on the basis of more extensive data. Case S. shows clearly that C.F. is not identical with the protein conditioning the formol-gel reaction nor with that which is precipitated by distilled water (D.F.). The property of cold precipitability seems to be not an acquired property of known protein fractions but the characteristic of a specific pathologic fraction.

Patient P., in contrast to Patient S., had a marked increase in blood globulin extending to the different fractions included in this term. The increase was marked even after the patient had achieved complete clinical recovery. An analysis carried out five months after recovery revealed a return of the total globulin content to practically normal and a negative formol-gel reaction but positive D.F. and C.F. tests.

Patient G. showed a similar trend. He had a clinical recovery; the serum protein value was at normal level; the formol-gel reaction, negative; the D.F., nil or traces; the euglobulin, normal or slightly increased. Nevertheless a C.F. was still clearly demonstrable six months after clinical recovery, although, of course, it was smaller after this time than during the heyday of the disease.

The clinical improvement is characterized by rise of albumin, fall of globulin and, particularly, euglobulin, and decrease of the pathologic fractions. The C.F. in particular may persist long after clinical recovery is considered to be complete. The question thus arises whether a patient whose serum is found to contain easily demonstrable pathologic protein fractions can be nevertheless regarded as completely cured. It is conceivable that the blood characteristics of such a patient are due to a condition of hyperstimulation of the reticulo-endothelial system.

Of thirteen cases of *Leishmania cutis* (Jericho rose) which came under examination, only 1 chronic case gave a positive C.F. test; it was graded 2.

C.F. in Dogs Infected With Kala-Azar.—Three further cases were examined—a mild, a medium, and a severe. Table II shows the results of the blood examinations as obtained when the dogs first came under examination.

TABLE II

CASE	TOTAL PROTEIN	ALBUMIN	GLOBULIN	EUGLOBULIN	C.F.	D.F.	FORMOL-GEL
1	6.69	2.44	4.25	0.69	0.32%	-	+ after 1 min.
2	10.06	2.63	7.43	2.45	0.37%	0.68%	+ after 1½ min.
3	12.38	2.00	10.38	5.47	3-4	1.1 %	+ instantaneously

TABLE III

DIAGNOSIS	TOTAL PROTEIN	ALBUMIN	GLOBULIN	EUGLOBULIN	C.F.	D.F.	FORMOL-GEL	REMARKS
Sepsis	6.23	3.47	2.76	Slight increase	3-4	+	-	Died
Malaria (chronic)	7.56	3.67	3.89	Increase	0.26%	+	-	Cholesterol, 160 mg. %
Malaria (chronic)	6.25	3.34	2.91	Increase	4	0	0	
Lepra	6.56	5.00	1.56	Normal	3-4	0	0	

The tabulated data corroborate that (except for the C.F. which is independent of all other fractions and seemingly so also of other elements making up the clinical picture) the tested globulin fractions parallel one another.

Other than in kala-azar, C.F. has been found positive in a few other cases of chronic infections. Table III describes the oustanding instances. The data in Table III also confirm that the C.F. is independent of the other globulin fractions.

Endocarditis Lenta

The condition of endocarditis lenta was selected for a special study since it seemed possible that C.F. in this condition might prove to be a character of diagnostic value. It should be emphasized in advance that the C.F. in cases of endocarditis lenta is meager as compared to the C.F. usually found in kala-azar and is only in exceptional circumstances accessible to quantitative estimation. It should be noted further that the precipitation is relatively sluggish and generally complete only after from three to four days. At 37° C. the sediment can, in all cases, be brought again into solution.

The impression is obtained that the C.F. increases if the disease becomes aggravated. Sulfanilamide in serum can inhibit or prevent the appearance of the C.F. As a comparison reference, eighteen sera from cases of endocarditis rheumatica were examined. Only one of them yielded a positive C.F. test, the precipitate obtained in this case being of a plastic irreversible type. In all the examined sera correlation between C.F., D.F., euglobulin, and formol-gel reaction was not in evidence. A summary of the findings is presented in Table IV.

Table IV

Endocarditis Lenta

DATE	PATIENT	TOTAL PROTEIN	ALBUMIN	GLOBULIN	EUGLOBULIN	C.F	D.F.	FORMOL-GEL
7/20	H.	6.3	3.4	2.9	1.5	1	0	After 1 hr. +
7/23		5.9	3.0	2.9	0.8	1	0	After 2½ hr. +
9/11		7.95	2.6	5.0	2.1	3	0	
9/18	Fir.	6.72	3.50	3.22	0.57	0	+	After ½ hr. +
10/19		7.82	3.10	4.72	1.62	3	+	After 1¾ hr. +
2/16	Lib.	8.98	3.11	5.87	3.4	2	+	
3/5		7.50	3.5	4.0	1.3	1-2	Trace	After 1 hr. +
3/30		6.8			1.1	3		
2/17	Saf.	9.1	4.6	4.5	1.7	3	0	
2/19		9.31	5.0	4.3	1.2	2	0	0
4/20	St.	8.16	3.3	4.9	Increase	3	+	
4/27		7.38			0.5	3	+	
1/12	Lech.	8.23	4.33	3.90	Increase	1.8%	+	Instantaneous +
2/12		8.55			Increase	2.5%	+	Instantaneous +
6/ 1	Brs.				Normal	3	+	0
6/ 8	Gl.	7.45	3.44	4.01	Increase	3	0	0
6/21		–	–	–	Increase	3	0	0

Although the amount of material examined so far is small, the possibility seems to be definitely indicated that presence of C.F. can have diagnostic significance in endocarditis lenta. Conclusive decision on this point must await the accumulation of more extensive data.

COLD PRECIPITATES IN SPECIAL SERA FROM CASES OF OTHER DISEASES (TABLE V)

In nephroses especially, serum samples frequently, although not always, become turbid in the cold, and after some time deposit a precipitate in the form of a ring near the surface. This can be redissolved at 37° C. rapidly and reappears if the serum is again cooled. Analysis of the fraction showed that it consisted largely of cholesterol and, in small part, of protein. The cholesterol level of the serum is not, however, determinant of the appearance of this cold fraction. In the so-called nephroses of pregnancy, positive C.F. tests have not been encountered.

It is pertinent to note that C.F. tests similar to those of nephroses have been recorded in three cases of morbus Gaucher and in one case of Niemann-Pick disease. Finally it may be mentioned that cold fractions, generally of an irreversible type, are encountered in the final stage of chronic uremia, in severe cirrhotic alterations of the liver, and also in cases of acute liver atrophy. Investigations designed to elucidate the nature of these cold fractions have been put under way. In all cases of this category, euglobulin was normal; the D.F., negative; and the formol-gel reaction, 0.

TABLE V

COLD FRACTION IN OTHER DISEASES

PATIENT	DIAGNOSIS	TOTAL PROTEIN	ALBUMIN	GLOBULIN	C.F.	UREA	URIC ACID	CHOLESTEROL
Ko.	Amyloid nephrosis; osteomyelitis	5.78	2.66	3.12	5	56	–	242
Sp. 1/18	Chronic nephritis and nephrosis	3.81	1.23	2.58	5	28	3.0	628
3/16	Chronic nephritis and nephrosis	4.46	2.55	1.91	3	52	4.4	1,000
5/4	Chronic nephritis and nephrosis	–	–	–	5	70	–	955
R.	Nephrosis; uremia	4.84	3.15	1.69	5	105	8.1	356
D.	Nephrosis 7/22	5.33	3.12	2.21	3	35	–	660
	8/7	4.57	1.44	3.33	5	32	–	635
B.	Chronic nephritis and nephrosis	5.13	2.38	2.75	3	143	–	386
L.	Nephrosis	4.95	3.10	1.85	1	–	–	515
F.	M. Gaucher	6.66	4.17	2.49	5	–	–	121
Z.	M. Gaucher	7.50	5.32	2.18	3 (Irreversible)	–	–	90
X.	M. Gaucher	–	–	–	5			125
W.	M. Niemann-Pick	6.54	3.48	3.06	5	–	–	219
R.	Chronic nephritis; uremia	7.40	3.60	3.80	4 (Irreversible)	315	10.0	(Xanthoprotein ++)
S.	Hypertension; uremia	7.23	4.40	2.83	4 (Irreversible)	200	6.8	(Xanthoprotein ++)
G.	Chronic nephritis; uremia	–	–	–	3 (Irreversible)	136	6.3	(Xanthoprotein +++)
L.	Cirrhosis, hepatic; hemachromatosis	6.08	3.17	2.91	3 (Irreversible)	21	–	285

HYPERGLOBULINEMIA AND PATHOLOGIC SERUM PROTEIN FRACTIONS IN PROTEIN HUNGER AND BLOOD-LOSS (TABLE VI)

Numerous investigations have revealed that in chronic protein hunger, blood loss, or plasmapheresis, either separately or combined, serum globulins

TABLE VI

DATE	TOTAL PROTEIN	ALBUMIN	GLOBULIN	EUGLOBULIN	C.F.	D.F.	FORMOL-GEL	BLOOD WITHDRAWN (C.C.)	HEMATOCRIT (HB.)	ERYTHROCYTES MILLIONS
					Dog L. (26 kg.)					
6/23	10.06	2.63	7.43	2.45	0.37	0.68	+ ($1\frac{1}{2}$ min.)	-	58 27	3.7
7/26	8.79	1.73	7.08	1.68	+ (None determinable)	0.86	-	-	55 29	4.08
8/ 4	10.94	1.71	9.23	4.69	0.50	1.13	+ (15 sec.)	360 600	58 28	4.25
8/ 5	9.56	1.05	8.51	5.06	1.13	0.56	-	75	43 26	3.11
8/ 6	10.63	1.32	9.31	5.88	2.50	0.69	Instantaneous	70	50 24	3.35
8/10	10.06	0.94	9.12	5.00	0.73	0.23	-		-	-
					Dog B. (21 kg.)					
5/24	12.38	2.0	10.38	5.47	+ (None determinable)	+ (None determinable) (1.1 5/27)	Instantaneous	-	78 30	6.0
6/12	11.63	1.68	10.45	4.63	+ (None determinable)	2.37	Instantaneous	220	65 26	5.1
6/26	11.63	1.63	10.00	5.88	1.00	1.00	Instantaneous	540	43 21	3.35
7/ 6	11.13	1.75	9.38	5.00	0.38	3.38	Instantaneous	590	38 20	2.19
7/13*	10.25	2.25	8.00	2.87	1.43	1.37	Instantaneous	120	54	2.75
7/15	9.38	2.63	6.75	2.00	+ (None determinable)	2.63	Instantaneous		-	2.97
7/17	9.56	3.16	6.40	5.56	+ (None determinable)	0.25	Instantaneous	60	55 29	2.78

*Meat diet from 7/13; condition—very poor.

are sustained at the normal level although serum albumins are decreased.[1] It appeared pertinent in view of our findings in kala-azar to establish whether the abnormally increased serum globulin fractions of this condition are affected by protein deficiency in the diet and by blood loss.

Two dogs (kala-azar) with marked hyperglobulinemia were selected for the purpose of these experiments and maintained on a protein-low diet.[4] At intervals of from two to three days blood was let from the animals in varying quantities.

Dog L. in the first stages of the experiment was reinfused with a suspension in saline of the removed erythrocytes (plasmapheresis). In a later stage reinfusion was omitted and the protein deficiency condition thus further enhanced. This treatment raises the need of hemoglobin neogenesis, which, according to Robscheit-Robbins and associates,[2] takes precedence over formation of other serum proteins. In the experiment with Dog. L. it is particularly evident that, despite marked protein deficiency in the diet and serious loss of blood, the total globulin, euglobulin, and the pathologic C.F. and D.F. continue their upward trend. In Dog B. the globulin level again was maintained and the pathologic fractions showed some increase. The experiments show that the greatly enhanced levels of globulin and pathologic protein are maintained despite chronic protein hunger combined with severe blood loss (Table VI).

It is clear that the production of this protein material is exclusively effected at the expense of endogenous materials and is entirely independent of dietary protein supply and of the protein reserves. On feeding of protein (meat) after prolonged protein hunger and after numerous blood lettings, the globulin values and the pathologic protein fractions of Dog B. were not varied, whereas the albumin values showed an immediate increase.

DISCUSSION

The data available suggest that cold labile globulin (or globulins) forms a pathologic fraction independent from other globulins and not necessarily parallel in trend with the latter. The salient characteristic of this fraction is its marked lability, a circumstance which renders electrophoretic investigation difficult or impossible. C.F. occurs most uniformly and in greatest abundance in kala-azar. Its possible diagnostic value in this condition, provided present experience is confirmed on a larger scale of material, has been indicated. The C.F. may also occur in other highly chronic infections, such as malaria and tuberculosis. Particular interest attaches to its occurrence in endocarditis lenta. It has been suggested that in the latter condition the C.F., although not abundant, may prove to be of diagnostic value. The form of distribution of the C.F. suggests that in chronic infections, which evoke strong defensive reactions, the sustained stimulation of the globulin-forming apparatus leads not only to increased formation of euglobulin, but also to the release into the blood of a serum-incompatible, particularly labile, globulin fraction. A C.F. of a very different nature is encountered in nephroses. In these conditions the C.F. consists largely of cholesterol but is nevertheless independent of the general blood cholesterol level. In conditions of uremia and liver cirrhosis in the final stage, an irreversibly precipitable C.F. is frequently encountered. The nature of this C.F. is still completely unknown. The experiments with dogs infected with kala-azar show that the enhanced production of globulin and the formation of pathologic globulin fractions in this condition is not dependent either on dietary supply of protein or on the protein reserves of the organism.

SUMMARY

The cold-precipitable protein found in patients with kala-azar (human and dog) forms an independent pathologic fraction separate in behavior and formation from other investigated globulins.

The C.F. can be demonstrated in the serum long after the patients are clinically recovered.

The manifold increase in the level of total globulin, euglobulin, the C.F., and the other pathologic fractions of patients with kala-azar are maintained despite chronic protein hunger and extensive blood loss. The production of the increased protein fractions is therefore independent of the dietary protein supply and of the body protein reserves. The presence of C.F. in cases of endocarditis lenta has been demonstrated and its possible diagnostic significance has been pointed out.

In severe lipoid nephroses, final phase of chronic uremia, and in severe cirrhotic alterations of the liver, C.F. of a peculiar nature occurs in the serum.

We are indebted to Prof. S. Adler of the Parasitological Department and to Dr. Tschernomoritz for their kind and generous help.

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LABORATORY METHODS

GENERAL

GENTIAN VIOLET-BLOOD AGAR PLATES USED IN AEROBIC AND ANAEROBIC CULTURES OF WOUNDS

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GENTIAN violet incorporated into media to produce selectivity of bacterial growth has been used for many purposes. Churchman[1] separated anthrax from a gram-negative rod and isolated each organism in pure culture by employing gentian violet and acid-fuchsin. Farley[2, 3] has used gentian violet as an acid in isolation of pathogenic molds. That gentian violet assists in isolation of the influenza bacillus was shown by Bernstein and Lowe.[4] This dye has also been used in bacterial counts of air, being employed in such studies by Thomas,[5] Thomas and Van den Ende,[6] Bourdillon, Lidwell and Thomas.[7] False presumptive tests for *B. coli* in water and milk analyses occur less frequently if gentian violet is incorporated into lactose broth than when lactose broth is used without an inhibiting agent. This work was done by Hall and Ellefson,[8, 9] who reported that the percentage of samples positive for *B. coli* was not lowered.

Petroff's medium for culture of the tubercle bacillus employs gentian violet in a concentration of 1:10,000 to discourage growth of molds and other common contaminants. The fact that acid-fast organisms, although gram-positive, are highly resistant to gentian violet, was first noted by Churchman[10] and has been elucidated by the work of Stearn and Stearn[11] who found that tubercle bacilli decompose gentian violet.

The dye, incorporated in blood agar plates, was used in a wound study reported by Francis,[12] who found in 300 swabs from burns and wounds inoculated in parallel on plain blood agar and gentian violet-blood agar that 32.7 per cent were positive for beta hemolytic streptococci when the dye was present, whereas, only 22.7 per cent were positive otherwise.

Gentian violet is known to be of definite aid in purifying anaerobes when their cultures are contaminated with *B. subtilis*, and has been used by Hall[13] routinely as an aid in isolation of anaerobes.[14] No reports, however, have been made of the numbers and types of anaerobes isolated in a series of cultures where gentian violet medium was compared with a dye-free medium.

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The work described in this report was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of Pennsylvania, and is part of the Contaminated Wound Project of the Subcommittee on Surgical Infections.

Received for publication, June 16, 1944.

The present work concerns 266 duplicate platings of either swabs or pieces of débrided tissue from wounds and burns cultured directly, or from platings of meat tubes previously inoculated with such swabs or tissue. The duplicate platings were made on 5 per cent blood plates of Difco's beef heart infusion agar with and without gentian violet in a final concentration of 1:500,000. About half of the platings were aerobic, the rest anaerobic. Identification of

TABLE I

ORGANISM	BLOOD AGAR PLATES ONLY	GENTIAN VIOLET-BLOOD AGAR PLATES ONLY	APPEARED ON BOTH
Bacillus subtilis and other gram-positive aerobic sporeformers	44	1	22
Bacterium aerogenes	1		3
Bacterium alkaligenes	1	11	7
Bacterium coli		1	12
Bacteroides melaninogenicus			2
Bacteroides (other than melaninogenicus)		2	1
Clostridium species			
Cl. bifermentans	1	1	6
Cl. botulinum Type A			1
Cl. capitovalis		3	
Cl. cochlearium	1	1	3
Cl. histolyticum		1	
Cl. non-fermentans	1		
Cl. paraputrificum		1	
Cl. putrificum		1	
Cl. sporogenes	3	2	8
Cl. subterminals		1	
Cl. tertium	1	1	2
Cl. welchii	1	4	8
Unidentified	3	5	6
New species	1		
Corynebacterium (aerobic)	14		7
Corynebacterium (anaerobic)	2	1	1
Eberthella enterica	1		
Flavobacterium group		3	3
M. candicans	1		
M. candidus			2
M. epidermidis	2		
M. nitrificans			2
M. ochraceus			1
M. tetragenus	3		
N. catarrhalis	1	1	
Proteus group			5
Ps. pyocyaneus			4
Sarcina lutea	1		
Staph. albus	1		5
Staph. anaerobic	7	12	21
Staph. aureus	89	6	30
Staph. epidermidis	6		3
Strep. aerobic nonhemolytic	8	13	46
Strep. aerobic alpha hemolytic		11	12
Strep. aerobic beta hemolytic	3	8	18
Strep. anaerobic nonhemolytic			3
Veillonella gazogenes		1	
Totals	197	92	244

SUMMARY

	AEROBES	ANAEROBES
Isolated on blood agar plates only	176	21
Isolated on gentian violet-blood agar plates only	55	37
Isolated on both	182	62

clostridia was done at the central laboratory of the Office of Scientific Research and Development wound-study project under the direction of Ivan C. Hall.

Table I shows that the total number of species isolated is greatly augmented by the duplicate culture on a gentian violet plate. On the whole, the results are in accord with the early studies of Churchman[10] on species susceptibility to the dye and the more recent work of Garrod[15] on the same subject. *B subtilis* was effectively inhibited; in the instances where it did occur on the gentian violet plates, its growth was sufficiently retarded to aid in isolating neighboring colonies.

In a study of the type here conducted, gentian violet agar could not be used as the only medium, since we were interested in isolation of the staphylococcus, which, next to *B. subtilis*, was the organism most consistently inhibited. In fact, appearance of organisms on gentian violet agar, when they were absent on plain blood agar, can largely be attributed to inhibition of the vigorously growing *B. subtilis* and the anaerobic sporeformers, and inhibition of staphylococci and diphtheroids, all organisms which may prevent growth of streptococci and other slowly growing bacteria. In the case of anaerobic cultures, gentian violet served the additional useful role of preventing the spreading of the motile clostridia, which also tend to monopolize the surface of an agar plate.

Gentian violet proved of more aid in isolation of *Cl. welchii* than the figures here would indicate. Early in the study it was found to be so useful that whenever stormy fermentation occurred in iron-milk, also used routinely for every specimen, the milk was plated only on a gentian violet-blood agar plate.

Since *Cl. tetani* was not isolated in this series, we cannot here answer the question of whether gentian violet would render more difficult the primary isolation of this organism. On the whole no indication was found that any one species of clostridium could not be readily isolated on gentian violet medium.

The large number of anaerobic staphylococci isolated are of interest for several reasons. They obviously were not affected by the concentration of dye used and their relatively high total incidence may be attributed to the fact that they grow out only after four to seven days, a time when the plain plates were often overgrown. Bergey's *Manual of Determinative Bacteriology*, 1939, lists only three species of anaerobic staphylococci and the majority of strains here isolated did not fit the description of any of the three.

Although complete plates were used as the duplicates in this study, similar results might have been obtained with the use of gentian violet spread over half the *surface* of a blood agar plate, as recommended by Fleming.[16]

SUMMARY

Blood agar plates containing gentian violet used in duplicate with blood agar plates without dye, permitted the isolation of many aerobes and anaerobes which would have been missed had the inhibiting medium not been employed. Such results appear to be due to the inhibition by gentian violet of the rapidly growing staphylococci and aerobic sporeformers, and to the fact that clostridia give less spreading overgrowth with this medium.

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POLYCHROME NO. 28

A SIMPLIFIED POLYCHROME STAIN FOR USE IN THE CLINICAL LABORATORY

CHARLES F. ELVERS

IN 1943 a preliminary report was published on a single process polychrome stain[1] designed primarily for use in staining spermatozoa and material encountered in specimens of prostate secretion. Later the stain was used routinely in the laboratories of the James Buchanan Brady Urological Institute of the Johns Hopkins Hospital to determine the presence or absence of bacteria, cells, mucin, lecithin, etc., and to differentiate between other normal and pathological materials. A considerable amount of this stain was also supplied to other institutions and laboratories. The numerous requests for reprints of this article received during the past year indicate that there is a definite need for a single process differential stain of this type, and experimental work was continued by modification of the original formula with the idea of simplifying the stain and adapting it for general use in the clinical laboratory.

In attempting to prepare a stain of this type, one must remember that while urine specimens are normally acid, prostatic secretion may show a hydrogen ion concentration varying from pH 6.5 to pH 8.0 or higher, and that dyes generally employed to demonstrate the presence or absence of leucocytes, epithelium, mucus, and most bacteria, fail to effectively stain fats, lecithin, spermatozoa and other seminal vesical elements. The miscibility of any compound stain with such a variety of material is of first importance.

The rate of acid formation in cytological material, as noted by Geschickter,[2] is more rapid than is generally supposed, and without some corrective measure uniformity of staining is not possible. In the past the technique in general use required a treatment by a pre-staining bath, either a mordant or buffer solution to reduce the thymonucleic and para-amino acids present as a result of cell degeneration. While this is effective as a control of the pH, its use imposes an additional and time consuming operation in the routine examination of clinical material, and except in research work is seldom carried out.

The simple or basic stain consisting of an aqueous-alcoholic solution of methylene blue has long been employed as a short cut in routine work. Simple basic stains of this type stain the cellular nuclei and most bacteria, but fail to differentiate between other cellular elements, and, therefore, have little value except to determine the number of bacteria present and their relation to the amounts of pus or exudate.

Polychrome No. 28, like the previous stain (No. 27) possesses the fundamental requirements of being a single process stain. It is rapid in action, produces minimal shrinkage of cells, requires no pre-staining baths, mordant,

From the Laboratories of John Wyeth and Brother Division, Wyeth Incorporated, Philadelphia, Pa.

Received for publication, April 14, 1944.

J. LAB. & CLIN. MED.
Vol. 29, No. 10, Oct., 1944

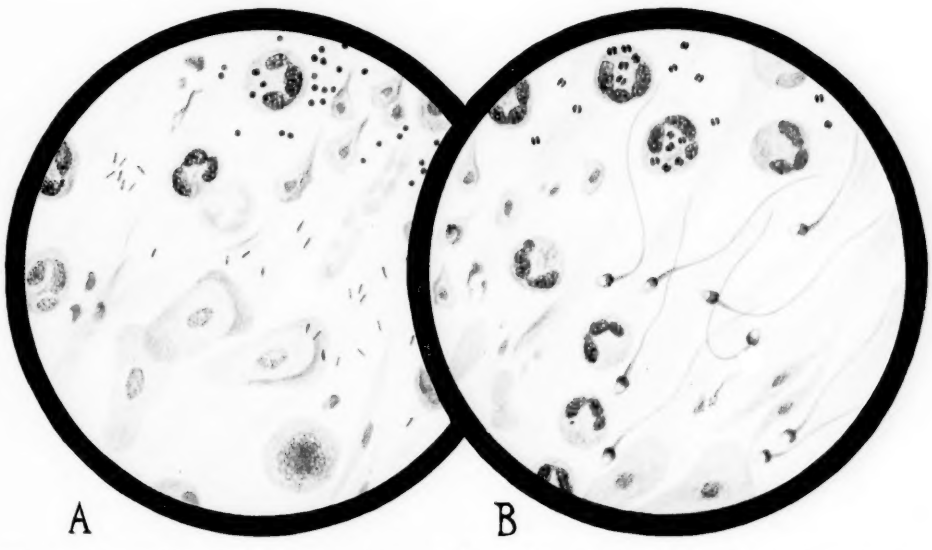

Fig. 1.—*A*, Centrifugalized urine specimen. *B*, Prostatic and seminal vesicle secretion. Stained by Polychrome No. 28, Elvers.

buffer, or decolorization and counter stain. The technique for its use is simple and completed in one action.

Delicate reactions such as those produced in the polychroming of Romanowsky and similar stains have been avoided, and in Polychrome No. 28 the differential staining reaction is effected by the use of a balanced formula of basic dyes in a buffered solvent. The neutrophilic, oxyphilic and basophilic elements react with that portion of the dye for which each has the greatest affinity, and this selective and contrasting color combination readily permits the identification of histological structures. Thin, even smears are essential for good staining and clear definition. Fresh material gives better results than that which has been allowed to dry for several hours, but slides which have been kept in a closed jar containing equal parts of 95 per cent ethyl alcohol, and of ether will stain well even after several weeks.

Freshly expressed prostatic secretion tends to become viscous on exposure to air. This viscosity precludes preparation of uniformly thin films, but if kept at oven temperature (56° to 58° C.) for ten to fifteen minutes the mucin will break down and the material may then be evenly spread on the micro slide.

The buffered solvent (stock) is made as follows:

Disodium phosphate	29 Gm.
Citric acid, C.P.	9.5 Gm.
Glycerin	40 ml.
Ethyl alcohol, 95 per cent	150 ml.
Distilled water q.s. to 1 liter.	

The pH of the above will be approximately 5.0, but may be measured potentiometrically and, if required, may be adjusted by adding small amounts of acid or disodium phosphate.

The stain is made by adding

2 Gm. of tetra-methyl thionin and

0.2 Gm. of rosaniline hydrochloride to

98 ml. of the pH 5.0 buffered solvent; stirring until all dye is dissolved; allowing to stand for twenty-four hours, and filtering or straining through cotton. Stain samples made by this formula still give sharp color contrast and good definition after eighteen months.

TECHNIQUE FOR USE

1. Fix thin, even smear to slide by heat or other method as desired, and cool to room temperature.

2. Cover smear with stain and allow to remain 30 to 60 seconds; slightly longer if deeper color is desired.

3. Rinse gently in distilled water, air dry and examine.

Mucin, cell cytoplasm, etc., are stained pink, nuclear material is blue. Cells which have lost their biochemical reaction due to degenerative changes are indefinitely stained a faint purplish red, the nucleus being darker than the cytoplasm. The front half of the head of spermatozoon remains clear or is

faintly stained, the back portion being dark blue, the spiral axillary thread and tail portion are red. Due to the fact that the tail structure is stained throughout its entire length, it appears much longer than when stained by other methods. Lecithin and cornified epithelial cells are bright red, bacteria are deep blue to purple.

Contrasting colors of the different elements are bright and well defined.

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SELF-BUFFERING STAINING SOLUTIONS*

With Special Reference to Wright's Polychrome Methylene Blue-Eosin Stain

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INTRODUCTION

POLYCHROME methylene blue-eosin stains have been widely used throughout the United States. Varying results, however, have been obtained in different laboratories employing the same staining solution.[1]

Kalthoff,[2] French,[3] Haynes,[4] Smith,[5] Conn and Margolena,[6] and Kingsley[7] suggested practical methods for obtaining uniform results with stains by the use of buffer solutions. McJunkin[8] demonstrated that much of the trouble encountered with polychrome stains could be eliminated if a phosphate buffer solution (pH 6.4) was used instead of distilled water in the staining procedure.

It was noted by the author that aqueous phosphate buffer solutions were subject to bacterial decomposition and that the addition of bacteriostatic substances in sufficient quantities to inhibit a biologic breakdown of the buffer solution, materially affected the efficiency of the Wright's staining solution. In an effort to offset the inconvenience of making buffer solutions at semimonthly intervals and effect an economy in reagents while maintaining staining efficiency, a study was made of the efficacy of buffering the methyl alcohol solution of Wright's stain.

EXPERIMENTAL

A. *Methyl Alcohol Buffer.*—Since Wright's stain is usually dissolved in methyl alcohol, it was decided to find methyl alcohol-soluble chemicals which were capable of buffering distilled water as commonly employed in the laboratory. Such a buffer system was found in sodium acetate and acetic acid.‡ These chemicals were readily soluble in methyl alcohol and/or in distilled water. The principal premise was that there would be practically no ionization when Wright's stain was dissolved in such a methyl alcohol-sodium acetate-acetic acid‡ solution. Ionization, however, would take place upon the dilution of the stain with distilled water and sufficient buffering quality would be released with the dissociation of the hydrogen ions in the aqueous solution.

Various concentrations and formulas based upon molar solutions of these chemicals were prepared which would give a pH of 6.4-6.6 (colorimetrically

*This research was begun at the Philadelphia College of Pharmacy and Science.

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‡Theoretically, acetic anhydride was considered in place of the acetic acid, but practically, a chemically pure grade of glacial acetic acid possesses insufficient water to materially effect ionization in the Wright's stain sodium acetate-acetic acid solution.

Received for publication, July 7, 1944.

and potentiometrically) when diluted with distilled water. The most suitable preparation of methyl alcohol buffer-Wright's stain solvent was as follows:

One hundred and twenty milligrams of $NaC_2H_3O_2 \cdot 3H_2O$ (sodium acetate—Merck's reagent) were dissolved in 100 c.c. of CH_3OH (methyl alcohol, free from acetone—Merck's reagent). To this was added 0.4 c.c. of a solution made by dissolving 0.1 c.c. of glacial acetic acid (Baker's C.P.) in 10 c.c. of methyl alcohol (Merck's reagent, free from acetone). The total amount of acetic acid in 100 c.c. of the sodium acetate-acetic acid-methyl alcohol solution is 0.004 c.c.

B. *Staining Solution.*—In every 60 c.c. of the methyl alcohol-sodium acetate-acetic acid solution, 0.1 Gm. of Wright's stain was dissolved. After 24 hours the staining solution was filtered. The buffering effect of this staining solution kept the pH approximately 6.4 to 6.6 when an equal volume of distilled water was added after the undiluted methyl alcohol-staining solution had first fixed the blood film.

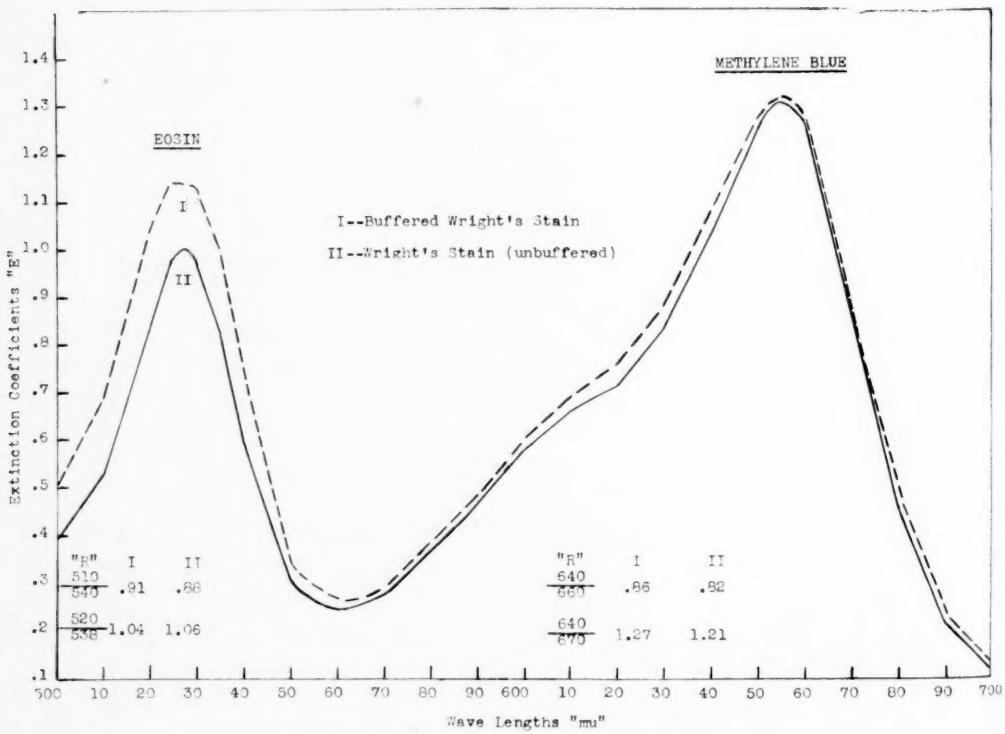

Fig. 1.—Extinction coefficient of Wright's stain.

C. *Staining Technique.*—Fifteen drops (about 0.35 c.c.) of Wright's stain were applied to the blood film and permitted to act for two minutes. Fifteen drops (about 0.75 c.c.) of distilled water were then added and three minutes were allowed for staining. The slide was rinsed in running water for 30 seconds, drained, and dried.

Comparison of Self-Buffered Wright's Stain With Nonbuffered Wright's Stain.—In November, 1938, sixteen different samples of commercially prepared Wright's stain were dissolved in duplicate sets; one series was made in buffered

methyl alcohol as discussed above (A and B), the other in methyl alcohol alone.

Numerous peripheral blood smears from normal individuals, as well as from patients suffering from malaria, filariasis, leprosy, leucemia, and pernicious anemia were prepared. Series of these blood smears were stained at monthly intervals for five years with the above staining solutions, employing distilled water as the diluent for the buffered stains and phosphate buffer solutions as the diluent for the unbuffered stains. In all instances the self-buffered Wright's stain solutions followed by the dilution with distilled water produced results comparable to the nonbuffered series. In not one instance has a buffered stain solution broken chemically or failed to stain properly. Final concentrations up to 300 mg. of sodium acetate and 0.01 c.c. of glacial acetic acid in 100 c.c. of methyl alcohol gave satisfactory results. An increase in the chemical concentrations, however, affected the erythrocytes from the cases of pernicious anemia. It was therefore deemed advisable to lower the chemical buffer concentration to a safe limit. This was 120 mg. of sodium acetate and 0.004 c.c. glacial acetic acid per 100 c.c. of methyl alcohol. In all instances the self-buffered stain solutions have resulted in good color detail. The differentiation of the cytoplasm of leucocytes and monocytes has been excellent as has been that of neutrophilic granules, eosinophilic granules, basophilic granules, blood platelets, and erythrocytes.

D. *Extinction Coefficient.*—Extinction coefficients were determined in the laboratory of the Biological Stain Commission in cooperation with the commission's technician, Mrs. A. P. Bradshaw in order to make certain that the stain itself was not altered by the buffering technique. The graphic results of the findings are presented in Fig. 1. The "extinction coefficients" of the eosin and methylene blue were not materially altered by the addition of the buffers to the dye solution. The changes in hue were so slight that no difference in staining due to this change would be expected.

SUMMARY AND CONCLUSION

A convenient and practical method for buffering methyl alcohol solutions of polychrome methylene blue-eosin stain is presented. The detailed method of preparation is described.

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A MECHANICAL DEVICE FOR MAKING MULTIPLE PARAFFIN RINGS FOR SLIDE PRECIPITATION TESTS FOR SYPHILIS

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IN A laboratory where a large number of slide precipitation tests for syphilis are run, the making of paraffin rings individually by hand is a tedious and time-consuming procedure. To eliminate these irksome factors the machine described here was devised. In the California State Public Health Laboratory, where this device was first put into operation, between four and five thousand Kline tests were done per day at the height of Selective Service activity. It required between five and six man-hours to make this many rings by hand, whereas with the machine that work can be completed in about 45 minutes.

The machine is based on the printing-press principle, with the desired number of paraffin rings being "printed" onto a glass plate in one operation. The size of the plates and the number of rings on each plate can be suited to individual requirements. We have found a plate 5 x 12 inches, accommodating 4 rows of 13 rings each, to be satisfactory. This provides for 50 tests together with positive and antigen controls. There is ample space for numbering.

The ring maker can be made for the most part of wood. The only metal required is as follows: about 4 feet of flat iron $\frac{3}{16}$ x $\frac{3}{4}$ inches, a metal rod $\frac{3}{4}$ inches in diameter by 16 inches long, and the metal paraffin pan.

The machine is shown in Fig. 1. The electric hot-plate *A* should be thermostatically controlled, but we have found an ordinary plate such as the General Electric Hotpoint (Cat. #40104) with three heat controls to be satisfactory. Maintaining the paraffin at the proper temperature is very important. This should be between 105°-110° C., at which temperature the paraffin smokes slightly. This temperature is held fairly well with the switch at "low."

The pan for the paraffin *B* can be made of 16 or 20 gauge galvanized sheet metal. The corner seams must be smooth to avoid leakage over the edge by capillary attraction. It is held in place by means of the spring clips *C*. The depth of the pan should be at least one inch and the length and width slightly greater than the corresponding dimensions of the printing head *M*.

The post *D* is made of $\frac{3}{4}$ inch round iron, and is about 16 inches long. It is fastened into the base *E* at the point of intersection of two lines perpendicular to the long axis of, and drawn from the mid-points of, the paraffin pan *B* and the platform *P*.

The swivel head *F* fits over the post *D* and is held in place by the platform *G* and the collar *H*. The stops *I* (there is one also on the opposite side, not visible) are driven into the platform *G* in the proper positions so as to allow the swivel motion through exactly 90°.

From the Division of Laboratories, California State Department of Public Health.
Received for publication, June 27, 1944.

The lever arms *J* and *K* are fastened onto the swivel head *F* as shown. The distances X-Y and X′-Y′ must of course be identical and will be between 7 and 9 inches depending on the size and shape of the glass plates used. The upper lever arm *J*, with the handle, should be about twice as long as the lower arm *K*. The brackets *L* fasten to the lever arms and screw into the printing head *M* as shown. The spring *S* must be quite strong to lift the heavy printing head.

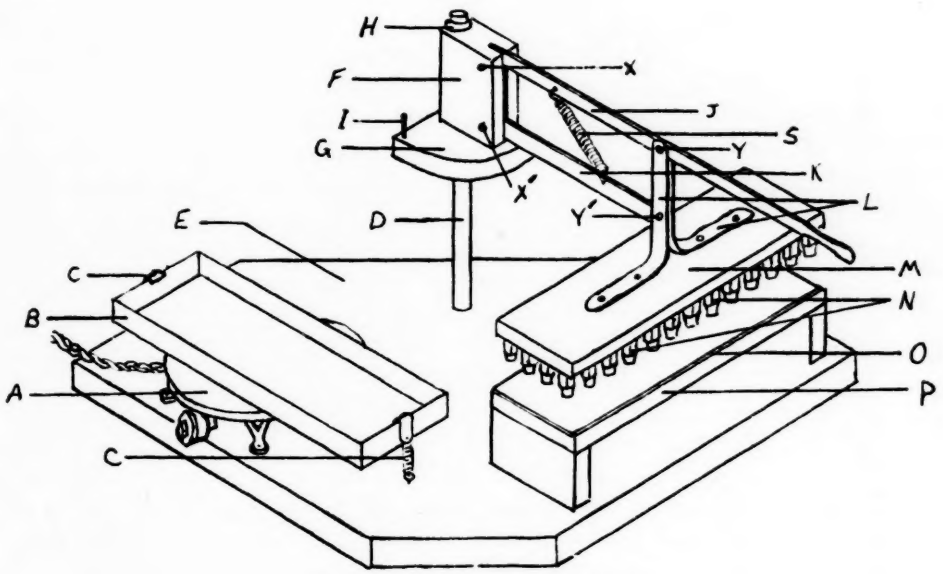

Fig. 1.—For explanation see text.

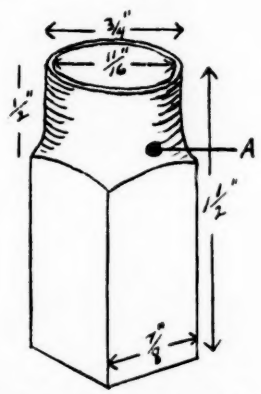

Fig. 2.—For explanation see text.

The printing head *M* is ¾ inch hard wood or 5-ply wood. It must not have any warp. The other dimensions depend on the size of glass plate used. The individual ring blocks *N* are fastened to it; these are described in detail below.

The platform *M* on which the glass plate *O* rests should be the same size as the platform *P*. This makes it easy to place the plate in exactly the same

position each time. The height of the platform must approximate that of the top edge of the paraffin pan.

The printing blocks *N* (enlarged in Fig. 2) are made of hardwood (oak or maple is suitable) and must be turned out on a precision lathe. The blocks are 1½ inches long by ⅞ inches square. An $^{11}/_{16}$-inch hole is first drilled into the exact center of the block to a depth of about ¾ inch. Then the outside is milled down to a diameter of ¾ inches leaving a tubular end with $^{1}/_{32}$-inch walls. This tubular part should not be more than ½ to ⅝ inches long to avoid warping. The use of square-shaped blocks also helps to prevent warping. Screw holes are drilled through the center of the square end of the blocks and fastened to the printing head *M* with a snug but not tight fitting screw. A ⅛-inch air hole *A* must be drilled through the wall as shown in Fig. 2.

The greatest difficulty was encountered in getting all blocks to print evenly and in eliminating "misses." This was "cured" by screwing all blocks tight, sanding the tubular edges on a large, even-surfaced mechanical sander, and then loosening the screws about half a turn to allow a little play for self-adjustment. The sandpaper used must be very fine to avoid tearing the thin walls. All burrs must be sanded off the tube edges as the final step.

SUMMARY

A mechanical device is described for making multiple paraffin rings used primarily in serological tests for syphilis such as those devised by Kline and Mazzini.

A similar device has been used in the California State Department of Health Laboratory for over a year and has been found to save considerable time and to produce more uniform rings than are ordinarily made by hand.

It is reasonably inexpensive, as it can be made largely of wood, but its construction requires some precision machinery and considerable mechanical skill.

A SIMPLE ACID WATER SOLUTION FOR BETTER VISUALIZATION OF MALARIAL PARASITES USING WRIGHT'S STAIN FOR THICK BLOOD FILMS

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CONSIDERABLE difficulty has been encountered in obtaining satisfactory differentiation of malarial parasites in thick blood films stained with Wright's stain. Either distilled water or buffered solutions containing monobasic potassium phosphate and dibasic sodium phosphate was used to dilute the stain and to wash it from the slide. The same technique, when applied to ordinary blood films, gave excellent cellular detail. Giemsa stain gives good differentiation of plasmodia in thick films, but since it is less commonly available than is Wright's stain, any alterations in technique which would permit the use of Wright's stain would have definite practical value. This note reports that good results may be obtained on thick blood films if: (1) the red blood cells are hemolyzed in a fixing solution rather than in water, and (2) an acid water is used to dilute the Wright's stain and to wash the slide.

The variations in techniques were made on thick blood films known to contain malarial parasites. Dilute acids, alkalies, and buffered solutions with a pH range of from two to ten were tried as diluents for the Wright's stain. An acid water, prepared by adding 0.06 c.c. (2 drops) 0.1 N HCl to 100 c.c. of distilled water gave excellent results. Clearer detail and less distortion of plasmodia were obtained if the blood was hemolyzed in a fixing solution rather than in water. The fixing solution was made by adding 1 c.c. of glacial acetic acid to 99 c.c. of 5 per cent formalin immediately prior to use. If the solution was kept in a covered staining jar, it was satisfactory for a large number of slides.

The dried thick blood film was immersed in the fixing solution for 10 minutes and then washed in two changes of distilled water for 5 minutes each time. After the slide had thoroughly dried in air, the film was covered with Wright's stain for 1 minute. An equal amount of acid water was then added and 10 minutes were allowed for staining. The slide was washed with acid water, dried, and examined.

With preparations treated in this manner, ring-form trophozoites show clearly against a dusky blue background. The cytoplasmic circle is a sky blue, while the chromatin dot is definitely red and easily detected under the microscope. Developing forms, schizonts, and gametocytes are also distinct.

From the Bacteriology Laboratory, U. S. Veterans Administration, Biloxi, Miss.

Published with the permission of the Medical Director, Veterans Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the author.

Received for publication June 19, 1944.

CHEMICAL

A MICRO-MINCER TO FACILITATE PREPARATION OF TISSUE EXTRACTS

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THE accurate determination of various biologically important substances in animal tissue depends largely on the degree of comminution of the sample, since intimate contact between the tissue and the extracting liquid is essential. The mortar and pestle are used to grind small tissue samples, but this method is laborious and quantitative transfers are difficult. Mechanical apparatuses have been proposed for this purpose by Corper and Cohn[1] and Potter and Elvehjem,[2] but these are somewhat different in principle from the apparatus herein proposed. The Waring Blendor, which is more similar in principle, is not adaptable to tissue samples of the size under consideration. The proposed micro-mincer is shown in Fig. 1.

Description.—The motive power for the mincer is supplied by a Precision "Aero-Mix" air-driven motor. Under actual operating conditions the speed is from 5,500 to 6,000 r.p.m. The shaft and cutting blades are made of chromium-plated steel. As shown in Fig. 1, the blades have no pitch, but two opposing blades are bent at an angle of about 30 degrees from the plane normal to the shaft. The end of the shaft is drilled and tapped and the blade is held in place by means of a chromium-plated bolt. The four leading edges of the blades are sharpened.

An ordinary round bottom 50 ml. centrifuge tube is used to hold the sample and extracting liquid. During the mincing operation the tube is held in a wooden block which fits snugly in the stand.

Operation.—From $\frac{1}{10}$ to 6 Gm. of tissue and 5 to 10 ml. of the extracting liquid are introduced into the 50 ml. tube. The tube is placed in the wooden holder and the holder raised to the stop. In this position the blade is approximately 0.5 inches from the bottom of the tube, and the holes in the stand and holder coincide. The metal pin is inserted to anchor the holder.

To avoid spattering, the mincing is begun with the motor running at slow speed. The speed is then increased to maximum and the mincing continued for from fifteen seconds to one minute. The motor is stopped, the pin removed, and the holder lowered so that the blade is clear of the mixture. The motor is now turned on full speed momentarily in order to throw any mixture adhering to the shaft and blade onto the side wall of the tube. The holder is lowered and the tube removed. When fibers are caught on the blade, it is very simple

From the Industrial Hygiene Research Laboratory, National Institute of Health, U. S. Public Health Service.

Received for publication, Aug. 15, 1944.

to remove them with a pair of forceps and place them in the tube with the minced tissue.

The shaft and blade are washed by placing from 5 to 10 ml. of the extracting liquid in another centrifuge tube and using the same procedure as that used for mincing the tissue, except that the motor is brought to full speed immediately and run for only a few seconds. The wash liquid is combined with the original mixture.

The extract may now be separated from the tissue by centrifuging in the same tube.

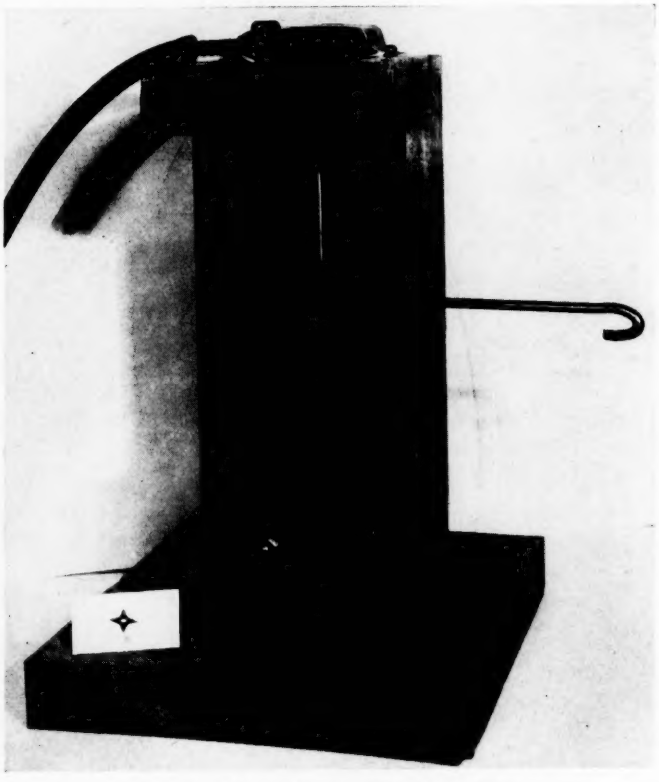

Fig. 1.—Micro-mincer with extra blade.

Use.—In the course of an investigation of the vitamin C content of guinea pig organs undertaken in this laboratory, considerable time and labor were saved by using the apparatus described herein for preparing trichloroacetic acid extracts. The kinds of tissues and approximate amounts of each used for these determinations were as follows: liver, 3.5 to 6.0 Gm.; kidney, 3.0 to 4.5 Gm.; spleen, 0.5 to 2.0 Gm.; adrenals, 0.1 to 0.3 Gm. Because of the small size of the adrenals, it was necessary to add a small amount of an inert material to the tissue so that the bulk of solids would be sufficient to insure satisfactory mincing. Paraffin shavings were found to be suitable for this purpose. Results obtained using this apparatus were in excellent agreement with those obtained when a mortar and pestle were used for grinding the tissues.

SUMMARY

A new micro-mincer is proposed to facilitate the preparation of extracts of small animal tissues. It is simple to construct and operates efficiently.

We wish to express our appreciation to Passed Assistant Surgeon Glen E. Ogden for determining the speed of the mincer.

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BOOK REVIEWS AND NOTICES

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